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(54) Title: METHOD FOR BASE SEQUENCING AND BIOLOGICALLY ACTIVE NUCLEIC ACIDS

(57) Abstract: Aptamers are nucleic acids and similar molecules, such as peptide-nucleic acids, that specifically bind to a ligand such as a protein or peptide. The present invention provides aptamers comprising at least one base capable of base pairing and different from the standard Watson-Crick bases. The present invention also relates to a method for preparation of such aptamers and to methods for sequencing nucleic acids that comprise at least one base capable of base pairing and different from the standard Watson-Crick bases.



**WO 02/44195 A2**

## DESCRIPTION

METHOD FOR BASE SEQUENCING AND BIOLOGICALLY ACTIVE  
NUCLEIC ACIDS

5

**Field of the invention**

The present invention relates to a nucleic acid comprising at least one base capable of base pairing and different from the standard Watson-Crick bases. The present invention also relates to a method for preparation of such a nucleic acid.

10 The invention also relates to a method for the sequencing of nucleic acids comprising at least one base capable of base pairing and different from the standard Watson-Crick bases.

**Background Art**

15 Aptamers are nucleic acids that specifically associate with a ligand. Aptamers can be selected *in vitro* by a technique known as SELEX (Systematic Evolution of Ligands by Exponential Enrichment). SELEX is a method for optimizing nucleic acids for high-affinity to given ligands starting from random sequence libraries (Hermann T. and Patel D.J., **February 2000**, *Science*, Vol.287:820-825; Tuerk C. and Gold L., **1990**, *Science*, Vol.249: 505).

20 Predominantly unstructured in solution, aptamers fold upon associating with their ligands into molecular architectures in which the ligand becomes specifically complexed with the nucleic acid. Because the evolutionary pressure on aptamer sequences during selection is directed primarily toward the binding of the ligands, the three-dimensional structures of aptamer complexes reflect highly optimized scaffolds for  
25 specific ligand recognition.

The architectures of aptamer complexes are valuable for the study of molecular recognition processes and yield a diversity of three-dimensional motifs, which recur in biologically relevant nucleic acid folds. It has been reported that small molecule RNA complexes are especially helpful for the rational exploration of RNA as a drug target.  
30 Drug design approaches for cellular RNA targets that combine structural data in RNA complexes with modelling techniques are especially promising, given the extraordinary success of molecular modelling of higher-order RNA architectures (Hermann and Patel,

2000). Nucleic acid aptamers provide unique tools in medicinal diagnosis and biotechnology and serve as therapeutics (Hermann and Patel, 2000). For example, aptamers that bind and inhibit human thrombin have been reported in Bock L.C. et al, 1992, *Nature*, Vol.355: 564-566.

5           However, there is a limitation in the use of aptamers because the structurally uniform four nucleotides are limited in possible alternative ways to pack around arbitrary ligands (Hermann and Patel, 2000).

          There is therefore the need in this field of science of new solutions for the availability of aptamers which allow the selection of a highly diverse and differentiated  
10   kind of ligands.

          The invention disclosed in the present application solves this problem in the art.

#### **Summary of the Invention**

          The present inventor has surprisingly found that nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard  
15   Watson-Crick (W-C) bases are particularly useful for the selection of new and specific ligands.

          Accordingly, the present invention relates to a method for the preparation of nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard Watson-Crick bases, wherein the standard Watson-Crick bases are  
20   adenine (A), cytosine (C), guanine (G), thymidine (T) and uracil (U).

          The method comprises:

- a) providing a specific, interesting ligand;
- b) synthesizing a pool of nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard Watson-Crick bases;
- 25   c) mixing this pool of aptamers with the specific ligand;
- d) selecting and amplifying a specific aptamer that binds to the specific ligand.

          Accordingly, the present invention also relates to a specific aptamer prepared as above.

          The present invention also relates to a method for recovering and determining a  
30   specific ligand comprising:

- i) providing at least one specific aptamer comprising at least one base capable of base pairing and different from the standard Watson-Crick bases;

ii) mixing this aptamer with a pool of ligands (for example human serum or proteins therefrom);

iii) recovering and determining the specific ligand bound to the specific aptamer.

According to another aspect of the present invention, the at least one aptamer according to the invention, can be fixed, directly or by means of a spacer sequence, to an insoluble substrate, for example a chip. The aptamer fixed on the substrate is then treated with a ligand mixture (for instance human serum) and the ligand bound to the aptamer is then recovered. According to a particular solution, the substrate comprises several specific aptamers according to the invention fixed on it. These specific aptamers fixed on a substrate are treated with a mixture of ligands and the ligands specifically bound to these aptamers are then recovered from the substrate. Preferably, the mixture of ligands is labeled and the quantitative amount of the ligand bound to aptamer can be determined.

Accordingly, the invention relates to a detection method for the detection of specific ligand, comprising:

- I) selecting at least one specific aptamer capable of binding to a searched specific ligand, the at least one specific aptamer being available in solution or fixed on an insoluble substrate ;
- II) mixing the at least one specific aptamer with a biological sample;
- III) detecting the presence and/or quantity of the searched specific ligand specifically bound to the at least one specific aptamer.

As a particular embodiment of the above method, the detection method can be a diagnostic method.

Accordingly, the present invention also relates to a detection or diagnostic kit comprising (i) at least one specific aptamer or (ii) one or more substrates having one or more specific aptamers fixed on it (them), and optionally one or more labels for labelling a ligand mixture.

Further, the invention relates to a pharmaceutical composition comprising the aptamer according to the invention in presence of a pharmaceutically suitable diluent, excipient and/or carrier.

According to another aspect, the invention relates to a method for sequencing nucleic acids comprising at least one base capable of base pairing and different from the standard Watson-Crick bases.

**Brief description of the drawings**

**Figure 1** shows an autoradiogram of DNA sequencing by the dideoxy method (Sanger) of Example 2. Column A) shows a sequencing reaction carried out under the standard conditions, wherein the concentrations of the non-standard W-C bases used as substrate (isoG and isoC), were the same as the standard bases (83.3  $\mu$ M). In B) the sequencing reaction was the same as A) except that the concentrations of isoG and isoC substrates were reduced to 40  $\mu$ M. iC and iG in Figure 1 represent isoC and isoG, respectively. The capital letter at the bottom represents its corresponding dideoxynucleotide comprised in the sequencing reaction. Figure 1 shows that when isoG and isoC are used and the concentration of isoG and isoC is lower than the standard W-C bases A, C, G, T (run B) the sequencing can be performed accurately.

**Figure 2** relates to the detection of a ligand protein in human serum using an aptamer chip as disclosed in Example 4. A) refers to the aptamer chip glass. A specific aptamer for the ligand protein having a base sequence disclosed in SEQ ID NO:4 and a negative control aptamer (SEQ ID NO:15) were fixed at locations 1 and 2, respectively. B) shows an autoradiogram of aptamer chip glass A after treatment with  $^{125}$ I-labeled human proteins.

**Figure 3** shows exemplary structural formulae of heterocyclic bases different from standard Watson-Crick bases ("non-standard bases"). R designates the point of attachment of the base to position 1 of a ribose or deoxyribose ring, X is either a nitrogen atom or a carbon atom bearing a substituent Z. Z is either a hydrogen, an unfunctionalized lower alkyl chain, or a lower alkyl chain bearing an amino, carboxyl, hydroxyl, thiol, aryl, indole, or imidazolyl group, Y is either N or CH, and each ring contains no more than three nitrogens consecutively bonded (according to Benner, S. A. US Patent 5,432,272).

Figure 4 shows the data from the individual electropherograms of the sequencing reactions in Example 5.

Figure 5 shows the composite electropherogram obtained by combining the data in Figure 4 to obtain the sequence of SEQ ID NO: 20.

**Detailed description of the invention**

According to a first aspect, the present invention provides a method for the

preparation of new nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard Watson-Crick bases. These new aptamers are particularly useful for the selection of new and specific ligands.

5 The preparation of aptamers according to the invention can be performed according to the SELEX protocol known in the art as described for example in detail in Tuerk & Gold, 1990, *Science* 249:505; Tuerk et al., 1992, *Proc. Acad. Sci. USA* 89:6988.

The ligand for aptamer selection can be any product useful as a ligand and able to be recognized by the aptamer according to the invention. Examples of ligands are reported in Hermann and Patel, 2000. The ligand can for example be an amino acid, a  
10 peptide, a protein, a lipid, an oligosaccharide, an alkaloid, a terpene, a co-enzyme, an antibiotic, or a derivative or complex of such molecules.

The ligand can be synthesized according to known techniques. In the case of a peptide or protein it can be produced on a peptide synthesizer or by recombinant DNA technology, or can be purified from a biological tissue.

15 The ligand can be a protein or a modified protein. For example the protein might be phosphorylated or non-phosphorylated, and/or glycosylated or non-glycosylated. The protein ligand can be produced according to the recombinant techniques known in the art, and can be made from a full-length cDNA library (for example using the methodology described in US Patent 6,143,528; in Carninci et al., **1996**, *Genomics*, 37:327-336;  
20 Carninci et al., **1997**, *DNA Research*, 4, 61-66; Carninci et al., **October 2000**, *Genome Research*, 1617-1630).

The ligand according to the invention is not limited by molecular weight, but preferably it has a molecular weight less than 5,000 daltons.

The pool of aptamers according to the invention comprising aptamers having at  
25 least one base capable of base pairing and different from the standard W-C bases can be prepared according to standard techniques known in the art, for example by an automated synthesizer, for example EXPEDITE 8909 using a standard protocol. Preferably, the pool synthesized is a random pool of single-stranded nucleic acids comprising a region of random sequence (which represents the random aptamer pool) flanked by defined regions,  
30 which will be recognized by specific primers for amplification of the template. The template generated can be preferably amplified, for example, using several PCR cycles. The PCR can be performed under standard conditions, however the concentration of the

triphosphate nucleosides can be modified according to the particular modified base different from a standard W-C bases. For instance, as reported in Example 3, when the bases different from the standard W-C bases used were isoC and isoG, the standard concentration of standard W-C base (A, C, G, T) was 200  $\mu$ M, while the isoC and isoG concentrations were 100  $\mu$ M.

Other amplification methodologies than PCR that are available in the state of the art can be used for the preparation of the template according to the invention. For example, LAMP (Loop-mediated Isothermal Amplification)(Notomi et al., **2000**, *Nucleic Acids Research*, 28:page e63); TMA (Transcriptional Mediated Amplification)(Kamisango, K. et al., **1999**, *J.Clin.Microbiol.*, 37:310-314); ICAN (Isothermal and Chimeric primer-initiated Amplification of Nucleic Acid)(TAKARA SHUZO CO, LTD, BioJapan **2000**, September 26-28); SDA methodology (Walker et al., **1992**, *Nucleic Acids Res.*, Vol.20:1691-1696).

The composition of the aptamer comprised in the template pool according to the invention will be as described below.

In step c) the aptamer pool according to the invention is mixed with the specific ligand prepared in step a).

The specific aptamer bound to the specific ligand is recovered and amplified (step d).

The nucleic acid aptamer, according to the invention, making up the pool of aptamers or pool of template comprising a region of random sequences of step b) is described as follows.

The aptamer is a nucleic acid comprising at least one base different from the standard W-C bases, wherein the standard W-C bases are defined as A, C, G, T or U. Preferably, the aptamer comprises at least one base different from the standard W-C bases and at least one standard W-C base.

This base different from the standard W-C bases can be any base capable of base pairing. It can be a non-standard W-C base as defined in Benner, S.A. US Patent 5,432,272, for example isoG and/or isoC (Roberts et al., **1995**, *Tetrahedron Letters*, Vol.36, No.21, pp.3601-3604, Elsevier Science Ltd), or can be a non-W-C base and capable of hydrophobic base pairing.

Examples of non-standard W-C bases are iso-C; iso-G; 2,6-diaminopyrimidine;

xanthine; 6-amino-5-substituted pyrazin-2(1H)-one;

1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione; 5-aza-7-deazaguanine;

6-amino-3-substituted pyrazin-2(1H)-one;

3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine;

- 5 2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one; other heterocyclic bases selected from the group consisting of the structural formulae of Fig. 3. In particular, the bases 2-amino-6-(N,N-dimethylamino)purine and pyridin-2-one are disclosed in Ishikawa, M. et al., **1999**, *Nucleic Acids Symp Ser*, 42:125-126.

- Examples of non-W-C bases (for instance exhibiting hydrophobic base pairing)  
 10 are 3-methyl isocarbostyryl; 5-methyl isocarbostyryl; 7-propynyl isocarbostyryl; m-xylene; 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylnaphthalene; 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl; 6-methyl-7-azaindole; 3-propynyl-7-azaindole; imidazopyridine; pyrrolopyridine. In particular, the base 7-propynyl isocarbostyryl, which is capable of pairing with itself, is described by Robert F.  
 15 Service in *Science*, **July 2000**, Vol.289:232-235.

- The aptamer comprising the at least one base capable of base pairing and different from standard W-C bases according to the invention shows a high variability of possible combinations. The nucleic acid aptamer according to the invention comprises at least one base capable of base pairing and different from the standard W-C bases,  
 20 preferably comprises at least one base capable of base pairing and different from the standard W-C bases and at least one standard W-C base (A, C, G, T or U). However, the number of bases capable of base pairing and different from the standard W-C bases is not limited. A nucleic acid aptamer according to the invention can therefore comprise, for example, 6 different bases, preferably A, C, G, T, isoG and isoC. However, it can also  
 25 comprise 8, 10, 12, 14 or more different bases (see US Patent 5,432,272).

- The use of bases capable of base pairing and different from the standard W-C bases according to the invention allows the preparation of specific aptamers having a higher variability than aptamers available in the state of the art. In fact, if we consider nucleic acids having different length, the number of different aptamers than it is possible  
 30 to prepare using the four standard W-C bases is much lower than the number of different aptamers that can be prepared using the bases different from standard W-C bases according to the invention, as reported in the Table 1 below. In Table 1, "4 W-C St. bases"



relates to a nucleic acid comprising only the W-C standard bases, "4 W-C st. + iG + iC" relates to a nucleic acid comprising the 4 standard W-C bases and 2 bases different from the standard W-C bases, for example isoG (iG) and isoC (iC).

5

Table 1

4 W-C st. bases      4 W-C st. + iG + iC

|    |        |           |            |
|----|--------|-----------|------------|
|    | 4 mer  | 256       | 1,296      |
|    | 6 mer  | 4,096     | 46,656     |
| 10 | 8 mer  | 65,536    | 1,679,616  |
|    | 10 mer | 1,048,576 | 60,466,176 |

As is clear from the Table 1, the variability of the aptamer disclosed by the present invention is a considerably improved compared to the aptamers available in the state of the art.

15

The aptamer according to the invention can be DNA, RNA or protein nucleic acid (PNA), and can be a single, double or triplex stranded nucleic acid. The aptamer can be of different lengths, preferably 12-300 bases.

The aptamer prepared according to the present invention can be used for recovering and determining a specific ligand from a pool of ligands (or a mixture of ligands) or from a biological sample. Such a method comprises:

20

- i) providing at least one specific aptamer comprising at least one base capable of base pairing and different from standard W-C bases, as above described;
- ii) mixing this aptamer with a pool of ligands or biological sample;
- iii) recovering and determining the specific ligand bound to the specific aptamer.

25

The at least one specific aptamer of step i) is prepared as above described according to steps a)-d). One specific aptamer can be used, or more than one different specific aptamers can be used at the same time, for instance if they are fixed on a insoluble substrate, for example a chip. Therefore, the aptamer can be used in solution or fixed or bound to an insoluble substrate, preferably fixed on a chip substrate.

30

The pool of ligands of step ii) can be any pool or mixture of ligands prepared with conventional methods known in the technique or can be a biological sample. The

ligand comprised in the pool of ligands can be preferably selected from amino acids, peptides, proteins, lipids, oligosaccharides, alkaloids, terpenes, co-enzymes, antibiotics, and their derivatives and their complexes.

When the ligand pool comprises proteins and/or peptides, they can be modified  
5 or non-modified, phosphorylated or non-phosphorylated, and/or glycosylated or non-glycosylated. Accordingly, the specific aptamer is able to distinguish between phosphorylated and non-phosphorylated, and/or between glycosylated and non-glycosylated and/or between modified and non-modified ligand proteins and peptides.

10 The ligands comprising the pool of ligands can be preferably labeled by contacting the pool of ligands with a label. The label can be any label able to be detected known in the art, for instance an isotope, chromophore or fluorophore label. Alternatively, a ligand can be detected by an antibody that specifically binds to the ligand.

In step iii) the ligand bound to the aptamer is detected and optionally separated  
15 from the aptamer and recovered. The detection can be either qualitative or quantitative. It is clear that in cases that more than one specific aptamer is used (for instance using a chip comprising several specific aptamers, different from each other, fixed on it) more than one ligand, each specific and bound to the specific aptamer, might be recovered. The selection can be done, for example, by detecting the label or labels introduced as above  
20 described. The detection, separation and recovery are performed according to the techniques known in the art.

As said above, the aptamer or aptamers according to the invention can be used in solution, or can be preferably fixed on an insoluble substrate, for example on a chip substrate. An insoluble substrate according to the invention can be any kind of surface of  
25 an insoluble substance, for example, beads, syringe, capillary, tube, plate. The insoluble substrate is preferably a chip (US Patent 5,525,464). The insoluble substrate can be used in a chromatographic format.

The aptamer(s) can be fixed to the insoluble substrate, preferably a chip substrate, according to standard techniques, for example synthesized on a chip as  
30 described by US Patent 5,837,832, or prepared and then fixed on a chip as described in EP 1041160. The aptamer can be fixed directly to the chip substrate or by means of a spacer or a linker sequence, for example as described in Example 4.

The aptamer fixed on the substrate is then treated with a ligand mixture (for instance human serum) and the ligand bound to the aptamer is then recovered and characterized. According to a particular solution, the chip has fixed on it several specific aptamers according to the invention. Preferably, the mixture of ligands is labeled and the  
5 quantitative amount of the ligand bound to aptamer can be determined.

Accordingly, the invention relates to a detection method for the detection of specific ligand, comprising:

- I) selecting at least one specific aptamer, according to the invention, capable of binding to at least one desired specific ligand, the at least one  
10 specific aptamer being available in solution or fixed on a substrate ;
- II) mixing the at least specific aptamer with a biological sample;
- III) detecting the presence and/or quantity of the at least one specific ligand.

As a particular embodiment of the above method, the detection method can be a diagnostic method for the detection of a ligand selected from a biological sample. An  
15 example of diagnostic methods according to the invention is a method for the detection of a disease, wherein the presence or amount of the specific ligand is related to a particular disease. The method can be used, for example, for detecting the concentration of cholesterol, testing for the drug concentration in the blood and other diagnostic utilities known in the state of the art. The ligand can also be, for example, a membrane receptor  
20 with a particular function or activity, the modification of which has influence on the expression of a particular disease.

The biological sample can be of any biological source e.g., vegetal or animal. For example, it can be a serum or a blood sample from a patient.

Accordingly, the present invention also relates to a detection or diagnostic kit  
25 comprising at least one specific aptamer according to the invention, or at least one substrate having one or more specific aptamers fixed on it, and optionally one or more labels for labeling the ligand pool or biological sample, as above described.

The specific aptamer according to the invention is particularly useful as disclosed for example in Hermann and Patel, 2000. For instance it can be used as a drug  
30 and for therapeutic treatment.

According to another aspect, the invention is a pharmaceutical composition comprising the aptamer according to the invention in presence of a pharmaceutically

suitable diluent, excipient and/or carrier. The invention also relates to a therapeutic method for the treatment of disease comprising the administration of the aptamer according to the invention, preferably in form of pharmaceutical composition as described above.

5           The use of aptamers for treatments in vivo is disclosed in literature (for example, Hicke et al., *The Journal of Clinical Investigation*, Oct. 2000, Vol.106, No.8; Watson et al., *Antisense Nucleic Acid Drug Dev.*, Apr.2000:10(2):63-75; and Floege et al., *American Journal of Pathology*, Vol.154, No.1, Jan.1999).

10           In Floege et al, in vivo effects of a nuclease-resistant high-affinity oligonucleotide aptamer were evaluated in a rat mesangioproliferative glomerulonephritis model. Twice-daily intravenous (i.v.) injections from 3 to 8 days after disease induction of a 2.2 mg/kg PDGF-B aptamer, coupled with 40-kd polyethylene glycol (PEG), led to a reduction of glomerular mitoses, a reduction of proliferating mesangial cells, etc.

15           In other experiments, Floege et al also observed that doses of PDGF-B aptamer as low as 2 mg total were sufficient for the treatment.

          Accordingly, the aptamer according to the present invention can be used for the preparation of a formulation as above described, to be administered in different routes, preferably by injection, comprising an amount of aptamer or aptamer substance  
20           according to the body weight and physiological conditions of the patient, however 2 mg/kg of body weight or more can be administered.

          The aptamer according to the invention can be sequenced.

          The inventor has found that the classical sequencing method known in the art, for example based on the Sanger method, (Sanger et al., **1977**, *Proc.Natl.Acad.Sci.USA*,  
25           74:5463-5467; Sanger et al., **1980**, *J.Mol.Biol.*, 143:161-178; Tabor and Richardson, **1989a**, *J.Biol.Chem.*, 264:6447-6458; Tabor and Richardson, **1989b**, *Proc.Natl.Acad.Sci.USA*, 86:4076-4080; US Patent 5,821,058) or the so-called transcriptional sequencing (TS) method described in US Patent 6,074,824, were not operable as such for base sequencing of a nucleic acid template comprising at least one  
30           base different from the standard W-C bases according to the present invention since terminators for bases different from W-C bases were not available in the art.

          The inventor of the present invention has therefore developed new and

improved methods for the determination of the nucleotide base sequence of nucleic acid templates comprising at least one base capable of base pairing and different from standard Watson-Crick (W-C) bases. According to one embodiment, the method comprises:

- 5           A) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- B) elongating said template using a primer or a promoter or a promoter and an initiator in the presence of a nucleic acid synthesizing enzyme, nucleic acid synthesizing enzyme substrates and substrate derivatives;
- C) determining the base sequence of the elongation product obtained in B).

10           According to one realization, the product of step B) can comprise a plurality of polynucleotides and each of said polynucleotides is separated from the others differing in length by a single nucleotide, and the nucleic acid sequence is determined according to method described in US Patent 5,821,058.

15           According to another realization, after step A) an annealing reaction between said template and a primer able to hybridize to said template is carried out.

20           The nucleic acid template according to step A) preferably comprises at least one standard W-C base A, C, G, T or U and at least one base capable of base pairing and different from the standard W-C bases. The nucleoside triphosphates having bases different from the standard W-C bases can be provided at the same concentration as those having the standard W-C bases. According to the nucleoside triphosphates having bases different from the standard W-C bases that are used, the nucleoside triphosphate concentration between the two kinds of bases can be different. When isoC and isoG are added, for instance, the concentrations of isoC and isoG triphosphates are lower than the concentration of the nucleoside triphosphates having the standard W-C bases.

25           The base different from the standard W-C bases can be any base capable of base pairing. It can be a non-standard W-C bases as defined in Benner, S.A. US Patent 5,432,272, for example isoG and/or isoC (Roberts et al., **1995**, *Tetrahedron Letters*, Vol.36, No.21, pp.3601-3604, Elsevier Science Ltd), or can be a non-W-C base (neither standard nor non-standard) and capable of hydrophobic base pairing.

30           Examples of non-standard W-C bases are iso-C; iso-G; 2,6-diaminopyrimidine; xanthine; 6-amino-5-substituted pyrazin-2(1H)-one; 1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione; 5-aza-7-deazaguanine;

6-amino-3-substituted pyrazin-2(1H)-one;  
3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine;  
2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one; other heterocyclic bases selected  
from the group consisting of the structural formulae of Fig. 3. In particular, the bases  
5 2-amino-6-(N,N-dimethylamino)purine and pyridin-2-one are disclosed in Ishikawa, M.  
et al., **1999**, *Nucleic Acids Symp Ser*, 42:125-126.

Examples of non-W-C bases (for instance exhibiting hydrophobic base pairing)  
are 3-methyl isocarbostyryl; 5-methyl isocarbostyryl; 7-propynyl isocarbostyryl;  
m-xylene; 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylnaphthalene;  
10 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl; 6-methyl-7-azaindole;  
3-propynyl-7-azaindole; imidazopyridine; pyrrolopyridine. In particular, the base  
7-propynyl isocarbostyryl, which is capable of pairing with itself is described by Robert F.  
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According to a particular realization, when the template is DNA, the  
15 synthesizing enzyme is a DNA-dependent DNA polymerase, the synthesizing enzyme  
substrate are dNTPs and substrate derivatives comprise ddNTPs.

Examples of DNA-dependent DNA polymerases are E.coli DNA polI Kelenow  
fragment (Sambrook, J. et al., **1989**, *Molecular Cloning: A Laboratory Manual*; Ausubel,  
F. M. et al. Eds., **1989**, In "*Short Protocols in Molecular Biology*," pp.201-231. Wiley,  
20 New York); modified T7 DNA pol (Sequenase) (Tabor, S. et al., **1987**, *Proc. Natl. Acad.*  
*Sci. USA* 84:4767-4771); and thermostable polymerases  
(Taq, Tbr, Tfl, etc.) (Eun, H-M., **1996**, *Enzymology Primer for Recombinant DNA*  
Technology, 1996 Academic Press).

Preferably, a dNTP or ddNTP used is labeled. According to a further particular  
25 embodiment, the primer or initiator of step B) is labeled. The label can be an isotope,  
chromophore or fluorophore label.

According to another particular realization, when the template is DNA, the  
synthesizing enzyme is a DNA-dependent RNA polymerase, the synthesizing enzyme  
substrates are NTP and substrate derivatives comprise 3'-dNTP derivatives.

30 Examples of DNA-dependent RNA polymerases are T7, T3 and SP polymerases  
(Axelrod, V. D. et al., **1985**, *Biochem.* 24:5716-5723; Parvin, J. D. et al., **1986**, *DNA*  
5:167-171).

The NTPs or 3'-dNTP derivative can be labeled. Preferably, the primer or initiator of step B) is labeled. The label can be a radioactive isotope, chromophore or fluorophore.

According to a further realization, when the template is RNA, the synthesizing enzyme is a RNA-dependent DNA polymerase, the synthesizing enzyme substrate is dNTP and substrate derivatives comprise ddNTPs.

Examples of RNA-dependent DNA polymerases are AMV and MoLV polymerases (Karanthanas, S., 1982, *Focus BRL*, 4:6-7; Geliebter, J., 1989, *Focus (BRL)*, 9:5-8; Hahn, C. S., et al., 1989, *Method Enzymol.*, 180:121-130).

The dNTP or ddNTP can be labeled. Preferably, the primer or initiator of step B) is labeled. The label can be an isotope, chromophore or fluorophore.

Further, when the template is RNA and the synthesizing enzyme is a RNA-dependent RNA polymerase, the synthesizing enzyme substrate is NTP and substrate derivatives are 3'-dNTPs derivatives.

An example of a RNA-dependent RNA polymerases is for example Q $\beta$  replicase (Kramer, F. R., 1978, *Proc. Natl. Acad. Sci. USA*, 75:5334-5338).

The NTP or 3'-dNTP derivative can be labeled. The primer or initiator of step B) can also be labeled. The label can be a radioactive isotope, chromophore or fluorophore.

The bases capable of base pairing and different from the standard W-C bases are preferably as described above.

An example of a non-standard W-C base capable of base pairing and different from the standard W-C standard bases is isoC and/or isoG.

2',3'-dideoxyisoguanosine 5'-triphosphate (ddisoG) and 2',3'-dideoxyisocytidine 5'-triphosphate (ddisoC) can be used as terminators in a process according to the present invention when isoG and/or isoC are used as substrate derivatives.

Accordingly, the present invention also provides new methods for the preparation of the compounds 2',3'-dideoxyisoguanosine 5'-triphosphate (ddisoG) and 2',3'-dideoxyisocytidine 5'-triphosphate (ddisoC).

The determination of the base sequence of nucleic acid templates according to the invention comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases, can also be performed by using MALDI-TOF-MS

analysis (US Patent 5,691,141)

Accordingly, the present invention also discloses a method for the determination of the base sequence of nucleic acid templates according to the invention comprising:

- 5                   A) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- B) elongating said template using a primer or a promoter or a promoter and initiator in the presence of a nucleic acid synthesizing enzyme, nucleic acid synthesizing enzyme substrates and substrate derivatives;
- 10                  C) determining the base sequence of the elongation product obtained in B) by determining the mass of fragmentation products using MALDI-TOF-MS analysis.

According to another embodiment, a method for determining the nucleotide base sequence of nucleic acid template comprising at least one base capable of base pairing and different from standard W-C bases can also be performed based on the chemical degradation method (Maxam-Gilbert method or improvements thereof). This method can be performed starting by chemical modification at only one end of a single strand template (Maxam and Gilbert, **1980**, *Methods Enzymol.*, 65:499-559; also Sambrook et al., 1989, Chapter 13.3), or by chemical modification of both ends of a double strand template (Maxam-Gilbert method (Maxam and Gilbert, **1977**, *Proc.Natl.Acad.Sci. USA*, 74:560-564).

A double strand template can be prepared from the single strand template of the invention according to standard techniques known in the art. For instance, a single strand template comprising at least one base different from W-C base is prepared, then a complementary strand is prepared by PCR amplification, using at least a PCR primer comprising a specific restriction enzymatic site (for instance FokI). The double strand template obtained from amplification is then cleaved using the specific restriction enzyme (for instance FokI: Toyobo Biochemical Catalogue) and the fragment obtained is chemically modified at both ends and sequenced by chemical degradation

30                  Accordingly, it is provided a method for determining the nucleotide base sequence of nucleic acid templates comprising at least one base capable of base pairing and different from standard Watson-Crick (W-C) bases, wherein standard W-C bases are



A, C, G, T or U, comprising:

- 1) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- 2) labeling one end of said template;
- 5 3) chemically degrading said labeled template;
- 4) determining the base sequence of the product obtained in 3).

Preferably, in the method above, the nucleic acid template comprises at least one standard W-C base A, C, G, T or U and at least one base capable of base pairing and different from the standard W-C bases.

- 10 The bases capable of base pairing and different from the standard W-C bases are preferably as described above.

An example of a non-standard W-C base capable of base pairing and different from the standard W-C standard bases is isoC and/or isoG.

- 15 The determination of the base sequence of nucleic acid templates according to the invention comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases can also be performed by using other technologies known in the art like, for example, pyrosequencing (real-time pyrophosphate DNA sequencing method) (Ronaghi, M. et al. *Anal. Biochem.* 242 (1996) 84-89; Ronaghi, M. et al. *Science* 281 (1998) 363-365), or array methods based on sequencing by hybridization
- 20 (Drmanac, I. et al. *Genomics* 4,1989, 114-128; also US 5,202,231).

Accordingly, the present invention discloses a method (pyrosequencing) for the determination of the base sequence of a nucleic acid template according to the invention comprising :

- a) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- 25 b) carrying out elongation by using a primer, a promoter, or a promoter and initiator in the presence of nucleoside triphosphates whose base comprises at least one base capable of base pairing and different from the standard W-C bases, by adding a first nucleoside triphosphate and detecting PPi release and
- 30 degrading the first nucleoside triphosphate, then adding a second nucleoside triphosphate and detecting PPi and degrading the second nucleoside triphosphate, and repeating the procedure according to the kind of base of the

nucleoside triphosphates provided;

- c) repeating the step above according to the template bases number desired to be sequenced;
- d) determining the sequence of the template as the reverse complement of the sequence of added nucleoside triphosphates;

The present invention also provides a method (hybridization sequencing) for the determination of the base sequence of a nucleic acid template according to the invention comprising :

- a) providing short (preferably 4-12 mer) oligonucleotides fixed on a chip, these oligonucleotides comprising at least one base capable of base pairing and different from the standard W-C bases and having overlapping frames displaced by one or two bases;
- b) hybridizing these oligonucleotides with a labeled template comprising at least one base capable of base pairing and different from the standard W-C bases;
- c) detecting the signal of the label;
- d) determining the sequence of the template by determining the set of overlapping oligonucleotides hybridizing to the labeled template.

The conditions for performing the hybridization sequencing are described in Drmanac, I. et al. *Genomics* 4,1989, 114-128 and also US 5,202,231.

The present invention will be further explained in more detail with reference to the following examples.

### **Example 1**

#### Preparation of 2',3'-dideoxyisoguanosine 5'-triphosphate (ddisoG)

Hydrogen peroxide (30 %, 3.5 ml) was added to a solution of sodium carbonate (2.6 g, 25 mmol) in water (25 ml). Maleic anhydride (2.45 g, 25 mmol) was then added, and the mixture was stirred at 0 °C for 30 min, at which point all of the maleic acid was dissolved. Concentrated sulfuric acid (1.5 ml) in water (7.0 ml) was then added at 0°C. The mixture was extracted with ether (8 x 25 ml), and the combined extracts were stored at 0°C.

A solution of monopermaleic acid in water was obtained by evaporating 0.31 ml of the ether extracts in the presence of water (36 µl) in a stream of air. The pH of the

solution was adjusted to 7.0 with NaOH (1 M), and a solution of disodium salt of ddATP (5 mg, 10  $\mu$ mol, ICN, in 18  $\mu$ l of water) was added. The reaction mixture was stirred for 24 h at room temperature, and the pH was adjusted to 4.5 with HCl (1 M). EtOH (absolute, 545  $\mu$ l) was added, and the resulting precipitate was recovered by centrifugation and  
5 dissolved in water (2.0 ml), the pH adjusted to 4.5, and the N-oxide of ddATP was obtained by precipitation with ether (3.6 mg, 73 %).

The N-oxide of ddATP (3.6 mg, 7.3  $\mu$ mol) was dissolved in water (4.5 ml), and the solution was placed in a photochemical reaction tube. The mixture was irradiated for 2 h with light from a high pressure mercury arc lamp, the pH adjusted to 10 (28 %  
10 aqueous ammonia solution), and the mixture was stirred at room temperature overnight.

Water was removed in vacuo, and the material was purified by HPLC (YMC ODS 120A semi-preparative column, eluted with 20 mM of aqueous triethylammonium acetate solution over 60 min in a gradient of 0.2 to 8 % acetonitrile).

Fractions containing product were collected, and the buffer was recovered in  
15 vacuo yielding 2',3'-dideoxyisoguanosine 5'-triphosphate.

Preparation of 2',3'-dideoxyisocytidine 5'-triphosphate (ddisoC)

Diethyl azodicarboxylate (40 % in Toluene, 1 ml, 2 mmol) was added to a mixture of 2',3'-dideoxyuridine (100 mg, 0.47 mmol) and triphenylphosphine (0.26 g, 1 mmol) suspended in tetrahydrofuran (1 ml). The mixture was stirred at room temperature  
20 overnight, and the resulting pale yellow suspension containing 2,5'-anhydro-2',3'-dideoxyuridine was used for the next reaction without purification.

Methanol (15 ml) was saturated with dry ammonia at 0°C, and the yellow suspension of 2,5'-anhydro-2',3'-dideoxyuridine was added. The mixture was stirred at room temperature for three days. TLC (silica gel, 20 % methanol/dichloromethane)  
25 showed essentially complete conversion. Stirring was continued for an additional five days, the solvents were removed by evaporation. The resulting residue was extracted with water (5 x 2 ml).

2',3'-deoxyisocytidine was obtained by evaporation of the solvent (48 mg, 48 %).

30 A solution of 2',3'-deoxyisocytidine (48 mg, 0.2 mmol) in trimethyl phosphate (0.822 ml) at 0°C was treated with phosphoryl chloride (0.039 ml) and stirred for 1.5 h. To the mixture was then rapidly added a solution of tris(tributylammonium)

pyrophosphate (1.2 mg) in dimethylformamide (2.5 ml). The mixture was agitated vigorously for 1 min.

A solution of triethylammonium bicarbonate (1 M, pH 8.0, 2 ml) was then added. The solvents were removed by evaporation, and the product was purified by HPLC  
 5 (YMC ODS 120A semipreparative column, eluted with 20 mM of aqueous triethylammonium acetate solution over 60 min in a gradient of 0.2 to 8 % acetonitrile).

## Example 2

### Sequencing of oligonucleotide

10 20 pmol of the reverse 3' primer, REV-4: 5'-AGC GGA TAA CAA TTT CAC AC-3' (SEQ ID NO:1)(synthesized with an EXPEDITE 8909 automatic synthesizer according to the standard protocol), was labeled using a MEGALABEL oligonucleotide labeling kit (Code No. 6070, Takara, Japan) and [ $\gamma$ -32P] ATP (Code No. AA0018, 6000Ci/mmol, Amersham Pharmacia Biotech, USA).

15 1.5 pmol of labeled primer prepared above and 0.75 pmol of an oligonucleotide template, PCR4-1: 5'-CAC GAC GTT GTA AAA CGA CGG CCA GTG TTA CGg cAT TGC cgA TGA CGA TGG TGT GAA ATT GTT ATC CGC T-3' (SEQ ID NO:2) (synthesized using an EXPEDITE 8909 synthesizer according to the standard protocol), and 2.25  $\mu$ l of 10x Klenow buffer (Code No. 6015A, Takara, Japan) was mixed and the  
 20 mixture volume adjusted to 18  $\mu$ l. The mixture was incubated at 95°C for 5 min, and put on ice. Three  $\mu$ l of water and 1.5  $\mu$ l of 2unit/ $\mu$ l Klenow fragment were added to produce the annealing mixture.

2  $\mu$ l of termination mixture, dddA (416.7  $\mu$ M dATP, 83.3  $\mu$ M dCTP, 83.3  $\mu$ M dGTP, 83.3  $\mu$ M dTTP, 40  $\mu$ M diGTP, 40  $\mu$ M diCTP, 100  $\mu$ M ddATP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), dddC (83.3  $\mu$ M dATP, 416.7 nM dCTP, 83.3  $\mu$ M dGTP, 83.3  
 25  $\mu$ M dTTP, 40  $\mu$ M diGTP, 40  $\mu$ M diCTP, 50  $\mu$ M ddCTP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), dddG (83.3  $\mu$ M dATP, 83.3  $\mu$ M dCTP, 416.7 nM dGTP, 83.3  $\mu$ M dTTP, 40  $\mu$ M diGTP, 40  $\mu$ M diCTP, 58.3  $\mu$ M ddGTP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), dddT (83.3  $\mu$ M dATP, 83.3  $\mu$ M dCTP, 83.3  $\mu$ M dGTP, 416.7 nM dTTP, 40  $\mu$ M diGTP, 40  
 30  $\mu$ M diCTP, 200 $\mu$ M ddTTP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), dddiG (83.3  $\mu$ M dATP, 83.3  $\mu$ M dCTP, 83.3  $\mu$ M dGTP, 83.3  $\mu$ M dTTP, 416.7 nM diGTP, 40  $\mu$ M diCTP, 100 $\mu$ M ddTTP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), dddiC (83.3  $\mu$ M

dATP, 83.3  $\mu$ M dCTP, 83.3  $\mu$ M dGTP, 83.3  $\mu$ M dTTP, 40  $\mu$ M diGTP, 416.7 nM diCTP, 100 $\mu$ M ddTTP, 16.7 mM Tris-HCl, pH 7.5, 166.7  $\mu$ M EDTA), was dispensed in 500  $\mu$ l microtube.

3.5  $\mu$ l of the annealing mixture was dispensed in each of the 6 tubes containing a different termination mixture. Each mixture was incubated at 37°C for 20 min. 4  $\mu$ l of stop solution (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was dispensed in each tube and incubated at 95°C for 3 min. After incubation, each tube was put on ice.

2  $\mu$ l of each mixture was applied on 20% polyacrylamide gel (20%T, 5%C, wherein T is acrylamide + bis-acrylamide, while C is bis-acrylamide) containing 7M urea with TBE buffer (88.9 mM Tris-borate, pH 8.3, 2.5 mM EDTA). Electrophoresis was carried out at 45 W constant power for 4 hrs.

After electrophoresis, the gel was fixed with 10% methanol and 10% acetic acid, for 15 min. The fixed gel was transferred to paper (3MM Chr, Whatman, UK), covered with wrapping film "Saran wrap" (Asahi Chemical Industries Co., Japan), and dried at 80°C for 1hr under vacuum.

The dried gel was exposed on a BAS imaging plate (Fuji film, Japan) for 1 hr, and then the image was detected using a BAS 2000 imaging analyzer (Fuji film, Japan).

In the experiment reported in Figure 1, it is shown that when isoG and isoC are used as substrates, when the concentrations of isoG and isoC as substrates are lower than the concentration of A, C, G, T, the sequence of the nucleic acid is legible. When instead the concentrations of isoC and isoG were the same that of A, C, G, T, the nucleic acid sequence could not be determined. However, when bases different from isoG and isoC (and from A, C, G, T or U) a substrate concentration the same, lower or higher than the A, C, G, T or U concentration can be used.

The sequence shown in Figure 1B), limited to the sequence comprising the included isoG and isoC bases (indicated as lower "g" and "c" letters in the sequence, and "iG" and "iC" in the Figure), is TTCACACCATCGTCATcgGCAATgcCG (SEQ ID NO:3).

### Example 3

#### Protein binding assays

The following experiment has been performed according to the SELEX protocol described in detail in Tuerk & Gold, **1990**, *Science*, 249:505; Tuerk et al., **1992**, *Proc. Acad. Sci. USA* 89:6988. In this experiment, the SELEX protocol was performed in a similar manner as for single stranded DNA.

#### Prerparation of target protein

A hypothetical protein from the RIKEN mouse full-length cDNA clone bank (<http://genome.rtc.riken.go.jp/>) was chosen as the target protein. The cDNA sequence of the selected clone is reported in **SEQ ID NO:4**. The open reading frame (ORF) is from bases 300-935. The sequence of the encoded protein (corresponding to the ORF) is reported in **SEQ ID NO:5**.

The ORF was amplified by PCR with the primers 5'-GTG CCC ACC TCC TCG GCA TAT GCC C-3' (**SEQ ID NO:6**) and 5'-TGA AGT TTC CAA TGG GAT CCT ATA AC-3' (**SEQ ID NO:7**). The amplified product was digested with *NdeI* and *BamHI*. The digested DNA was ligated into the corresponding sites of the vector pET16b (Novagen, 69662-3, USA). The recombinant plasmid was introduced into *Escherichia coli* BL21(DE3). The transformant was cultivated in LB medium containing 100 µg/ml of ampicillin. When the optical density at 600 nm reached 1.0, 100 mM isopropyl-β-D-thio-galactoside (IPTG) was added to the final concentration of 0.4 mM. After cultivation for 3 hrs, the *E. coli* cells were collected by centrifugation at 5,000 x g for 5 min at 4°C. The harvested cells were resuspended in 20 mM phosphate buffer (pH 7.4), sonicated, and centrifuged at 15,000 xg for 30 min at 4°C. The supernatant was applied to a nickel-chelate column (HisTrap kit, Code No. 17-1880-01 Amersham Pharmacia Biotech, USA). The absorbed protein was eluted with 300 mM imidazole. The purity of the expressed protein was tested by SDS-PAGE. The yield of expressed and purified protein was 50 µg/l culture.

#### Preparation of random single stranded DNA pool

A random template pool of single stranded DNA that consists of a 40 nucleotide random region (defined as [N40]) flanked by a left flanking region 5'-TGT AAA ACG

ACG GCC AGT-3' (SEQ ID NO:8) and by a right flanking region 5'- GT GTG AAA TTG TTA TCC GCT-3' (SEQ ID NO:9) was synthesized using an automated DNA synthesizer (EXPEDITE 8909 using the standard protocol).

The random template pool of single stranded DNA is therefore defined in this way:

5' TGT AAA ACG ACG GCC AGT-[N40]-GT GTG AAA TTG TTA TCC GCT3'.

The random pool region [N40] was generated by reacting with all six types of phosphoramidites (that is A, T, C, G, isoC and isoG) at the fixed concentrations of 5%(w/w) in acetonitrile 95%.

Defined nucleotide sequence primers (SEQ ID NO:1 and SEQ ID NO:10) for the flanking regions of [N40] of the template pool served as primer annealing sites for PCR. The complexity of the template pool was estimated as in the order of  $10^{14}$  molecules. After the synthesis, the template pool was amplified by PCR with the corresponding primers 5'-AGC GGA TAA CAA TTT CAC AC (SEQ ID NO:1) and 5'-TGT AAA ACG ACG GCC AGT -3' (SEQ ID NO:10) under standard PCR conditions except the concentration of isoG and isoC (100  $\mu$ M each) corresponded to one-half of the concentration of the other dNTPs (dATP, dTTP, dCTP and dGTP) (200  $\mu$ M each for A, C, G, T). The above obtained DNA was used for a second PCR, without adding the 3' primer and, a single stranded DNA was generated. 1  $\mu$ M oligonucleotide having the sequence 5'-GT GTG AAA TTG TTA TCC GCT-3' (SEQ ID NO:11) which was complement of the 3' primer, was added to inactivate the remaining 3' primer and maximize the fraction of DNA synthesis producing random single stranded DNA resulting from extension of the 5' primer.

#### Binding assay

The single stranded DNA pool (200pM) prepared above was incubated with the protein (obtained in the preparation of target protein above) in a binding buffer of PBS (10mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8mM KH<sub>2</sub>PO<sub>4</sub>, 130mM NaCl and 2.7mMKCl, pH7.4) containing 0.01% human serum albumin (SIGMA, product No. A1653) and 1mM MgCl<sub>2</sub>. The mixture of the protein and the random single stranded DNA pool was incubated at 4°C overnight, and then incubated at 37°C for 15 minutes. Then the obtained protein-DNA complex was separated from the unbound DNA and protein species by nitrocellulose

filter partitioning methods as described in Jellinek et al., **1994**, *Biochemistry*, 33:10450-10456. Nitrocellulose filters (Millipore, 0.45  $\mu$ m pore size, type HA) were washed with 5ml PBS buffer before using for selection. The mixture of the protein and the random single stranded DNA pool was applied to the filters under gentle vacuum in 45  $\mu$ l aliquot and washed with 5ml PBS. The selected single stranded DNA bound to the protein was then extracted from the filters, separated from the protein (according to Jellinek et al., 1994) and amplified again by the PCR methods described above. This cycle was repeated 20 rounds. The selected single stranded DNA, which is indicated as Template 1, comprises an oligonucleotide aptamer sequence. It was sequenced according to the method described in Example 2. The sequence of Template 1 is 5' TGT AAA ACG ACG GCC AGT GCG TAA CgG GGT cTA TGT TCC CGC ACA CcG TGG CAA AAC TGT GTG AAA TTG TTA TCC GCT 3' (**SEQ ID NO:12**). The sequence of the aptamer portion is 5'-GCG TAA CgG GGT cTA TGT TCC CGC ACA CcG TGG CAA AAC T-3' (**SEQ ID NO:13**).

#### Example 4

##### Aptamer Chip analysis

##### Aptamer preparation

Template 1 (**SEQ ID NO:12**) comprising the aptamer (**SEQ ID NO:13**) selected and sequenced in Example 3, was amplified by a standard PCR method with a primer having **SEQ ID NO:10** and a primer 5'-TGC CAT TTC ATT ACC TCT TTC TCC GCA CCC GAC ATA GAT GAC ACT ACT ACG GTA TGA TCC TAT GGA GAA CGC TCA GCG GAT AAC AAT TTC ACA C-3' (**SEQ ID NO:14**) which comprises the recognition site of *Pf-SceI*, a spacer of 50 bp 5'-GCA CCC GAC ATA GAT GAC ACT ACT ACG GTA TGA TCC TAT GGA GAA CGC TC-3' (**SEQ ID NO:15**), and the annealing site to single stranded DNA 5'- A GGG GAT AAC AAT TTC ACA C-3' (**SEQ ID NO:16**), and in the standard condition (200  $\mu$ M for A, C, G, T) except for the concentrations of isoG and isoC which were 100  $\mu$ M.

The purpose of the spacer sequence was to bind the aptamer sequence to a chip substrate.

After PCR, the obtained double stranded DNA comprising the aptamer (this double stranded DNA being defined Template 2) was incubated at 37°C for 30minutes



with *Pf-SceI* (New England BioLabs) to create an overhanging 3' end.

#### Preparation of aptamer chip

5 An amino group was introduced into the 3' end of the synthesized Template 2 (double stranded DNA) as follows.

1  $\mu$ g of the above Template 2 was incubated at 37°C for 1 hour with 50 units of terminal deoxynucleotidyl transferase (TdT)(TOYOBO Japan) in 50  $\mu$ l TdT buffer containing 0.5M 2', 3'-deoxy-5-(3-aminopropynyl)UTP, and the DNA was collected by ethanol (EtOH) precipitation by using for 1 volume of reaction mixture 1/10 volume of  
10 3M CH<sub>3</sub>COONa and 2.5 volumes of EtOH.

The substrate of the aptamer chip was made from a glass slide (S12E 3x1", 0.93 to 1.05mm). The slide was immersed in 100% trifluoroacetate at room temperature for 1 hour and dried. Then it was immersed again in 2%(v/v) aminopropyltriethoxysilane and 50% (v/v) acetone at room temperature for 24 hours following by washing with 50% (v/v)  
15 acetone three times and with 100% acetonitrile one time and then dried.

0.5  $\mu$ g of Template 2 (double stranded DNA) with the amino group at 3' end and succinic anhydride at the final concentration of 5% were allowed to react in a volume of 10  $\mu$ l to introduce a carboxy group to the 3' end of the DNA. This solution was mixed and immediately used for ligation to a slide glass.

20 Template 2 with the carboxylated 3' end was fixed on the aminated glass slide as follows. Template 2 with carboxylated 3' end was mixed with carbodiimide at a final concentration of 5% and 0.4  $\mu$ l of the mixture was dotted with a micropipette on the aminated glass slide. The glass slide was then incubated at 50°C for 6 hours, and washed with a solution of 10mM Tris-Cl [pH8.0], 1mM EDTA, 0.1% SDS, and 0.1M NaOH  
25 followed by washing with a solution of 10mM Tris-HCl [pH8.0], and 1mM EDTA. Thus, the glass slide on which single stranded DNA was fixed was obtained. It consists of a single stranded DNA comprising a primer sequence, the aptamer and the spacer. The DNA is fixed on the chip substrate by the spacer sequence.

#### 30 Detection of target proteins in human serum

The glass slide (3 x 1", 0.93 to 1.05mm) on which single stranded DNA is fixed (aptamer glass prepared above) was pre-treated by incubating in PBS containing 3%

gelatin (Bio-Rad) at 37°C for 10 min, and maintained in PBS solution. Human serum (SIGMA, products No. S7023) was diluted (to 0.1 mg protein/ml) with PBS, and labeled with <sup>125</sup>I-IODOGEN (Pierce) to a specific activity of 70 mCi/mg protein. 0.5ml solution of <sup>125</sup>I-labeled serum protein was put on the aptamer glass which was pre-treated with PBS  
 5 containing gelatin, and incubated at 37°C for 30 min. The aptamer glass was then washed gently 10 times with fresh PBS containing 3% gelatin (Bio-Rad) at room temperature. The radioactivity on the aptamer glass was detected by a conventional autoradiographic method.

Figure 2 shows the detection of a ligand protein in human serum using the  
 10 aptamer chip as disclosed in this Example 4. A) refers to the aptamer chip glass. A specific aptamer for the ligand protein having a base sequence disclosed in SEQ ID NO:4 and a negative control aptamer 5'- TGT AAA ACG ACG GCC AGT TTC CgG AGT CAC gGC TGC GGG cCG TCT GAG CCG TTT GCA CGT GTG AAA TTG TTA TCC GCT -3' (SEQ ID NO:17) were fixed at locations 1 and 2, respectively. B) shows an  
 15 autoradiogram for aptamer chip glass A after treatment with <sup>125</sup>I-labeled human proteins.

### Example 5

#### Sequencing of oligonucleotide using 6 fluorescent dyes

Six labeled with fluorescent dyes primers were prepared for sequencing. Four  
 20 fluorescent dye primers were commercial products (sold as a set of four dyes), BODIPY dye M13 forward primer (catalog number : 5016-FPW, SeqWright Inc., US ; Metzker, M.L., Lu, J., Gibbs, R.A.: Electrophoretically Uniform Fluorescent Dyes for Automated DNA Sequencing. *Science*, 271, 1420-1422, 1996). The other two fluorescent dye M13 forward primers were obtained from Genset: one was BODIPY-630 /650 (absorption  
 25 maxima : 625nm, emission maxima : 640nm) and the another was BODIPY-TR (absorption maxima : 589nm, emission maxima : 617nm). These two fluorescent dye M13 primers comprised the BODIPY-630/650 or the BODIPY-TR dye (produced by Molecular Probes Inc.) attached to the 5'-end of the M13 forward primer having the sequence 5'-GTA AAA CGA CGG CCA GT-3' (SEQ ID NO:18) (produced by Genset).  
 30 Six micro tubes (MicroAmp Reaction tubes, catalog number N801-0533, Applied Biosystems) were prepared for dye primer sequencing reactions. In each tube, 2 µl of a mixture comprising 0.125 pmol of each fluorescent dye primer, 40 fmol of an

oligonucleotide                      template                      5'-

CTATGACCATGgTcTCGCCTTGgTcTTTAGGTGACACTATTTTACTGGCCGTCGT  
TTTAC -3' (SEQ ID NO:19) [“g” indicates diGTP, (deoxy isoGTP) and “c” indicates

diCTP (deoxy isoCTP)] synthesized using an EXPEDITE 8909 automatic synthesizer according to the standard protocol, and water were mixed with 2  $\mu$ l of reaction mixture (1U Thermo Sequenase DNA polymerase; 150  $\mu$ U *Thermoplasma acidophilum* inorganic pyrophosphatase; 20 mM Tris-HCl, pH 8.5, 1mM dithiothreitol, 100mM KCl, 0.1mM EDTA, 0.5% Tween-20, 0.5% Nonidet P-40, 50% glycerol, 65mM MgCl<sub>2</sub> ) and with 4  $\mu$ l of each termination mixture to form a final mixture of 8  $\mu$ l. The contents of the termination mixtures were:

d/ddA mixture : 30  $\mu$ M dATP, 150  $\mu$ M dCTP, 150  $\mu$ M dGTP, 150  $\mu$ M dTTP, 150  $\mu$ M diGTP, 150  $\mu$ M diCTP, 150  $\mu$ M ddATP;

d/ddC mixture : 150  $\mu$ M dATP, 30  $\mu$ M dCTP, 150  $\mu$ M dGTP, 150  $\mu$ M dTTP, 150  $\mu$ M diGTP, 150  $\mu$ M diCTP, 150  $\mu$ M ddCTP;

15 d/ddG mixture : 150  $\mu$ M dATP, 150  $\mu$ M dCTP, 30  $\mu$ M dGTP, 150  $\mu$ M dTTP, 150  
 $\mu$ M diGTP, 150  $\mu$ M diCTP, 150  $\mu$ M ddGTP;

d/ddT mixture : 150  $\mu$ M dATP, 150  $\mu$ M dCTP, 150  $\mu$ M dGTP, 30  $\mu$ M dTTP, 150  $\mu$ M diGTP, 150  $\mu$ M diCTP, 150  $\mu$ M ddTTP;

d/ddiG mixture : 150  $\mu$ M dATP, 150  $\mu$ M dCTP, 150  $\mu$ M dGTP, 150  $\mu$ M dTTP,  
20 30  $\mu$ M diGTP, 150  $\mu$ M diCTP, 150  $\mu$ M ddisoGTP;

d/ddiC mixture : 150  $\mu$ M dATP, 150  $\mu$ M dCTP, 150  $\mu$ M dGTP, 150  $\mu$ M dTTP,  
150  $\mu$ M diGTP, 30  $\mu$ M diCTP, 150  $\mu$ M ddisoCTP;

The final mixture of each tube was reacted using a thermal cycler ( DNA Engine Tetrad thermal cycler, which is a thermal cycler having 4 independent thermal cycling blocks, and is a trade name of MJ Research Inc. US) according to the standard protocol for Thermo Sequenase DNA polymerase (Amersham Pharmacia Biotech Ltd.). A description of the use of the cycle sequencing program of DNA Engine Tetrad for Thermo Sequenase is disclosed in “Techniques of genome analysis in large-scale and gene functional analysis in the post-genome era”, Hayashizaki et al., Publisher: Nakayama-shoten Co., Ltd, 2001, Japan. The reacted samples were transferred to 0.5 ml tubes (GeneAmp Thin-Walled Reaction tubes with Flat Caps, catalog number N801-0737, Applied Biosystems), then precipitated with EtOH according to the following procedure.

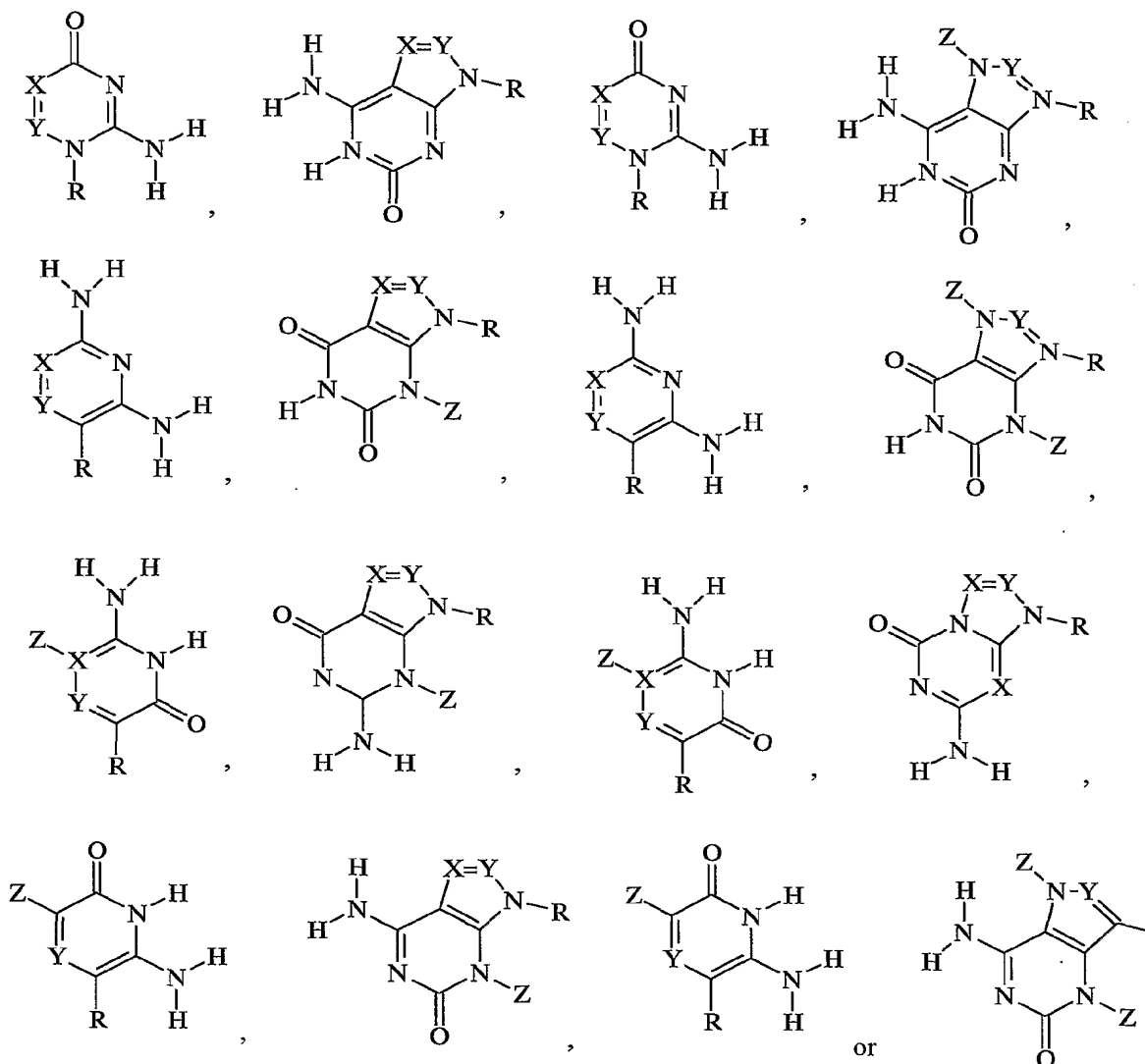
16  $\mu$ l of 99% EtOH and 0.8  $\mu$ l of 0.3M CH<sub>3</sub>COONa solution were added to each tube and the tube was centrifuged at 15,000 rpm for 30 min. at 4°C. After removing the supernatant, 200  $\mu$ l of 70% EtOH were added to each tube and the tube was centrifuged at 15,000 rpm for 10 min. at 4°C. After removing the supernatant, the tubes were left upside down for 5 minutes at room temperature in order to let water and EtOH evaporate. The samples were dissolved in 4  $\mu$ l of water per tube and mixed. After drying up at 65°C, each sample was dissolved in 4  $\mu$ l of 2  $\mu$ M EDTA solution, transferred to a 96-well plate ( catalog number 21971, Sorenson BioScience Inc. ), incubated at 95°C for 5 min, and put on ice.

Electrophoresis and fluorescence detection of reaction products were performed using the SCE 9610 Genetic analysis system (SpectraMedix Co. US) according to the manufacturer's procedure. After smoothing by using Fourier transformation and low pass filtration functions, background subtraction was performed for all data. For each 5 peak interval, a local minimum point, which value was minimum in the interval, was selected and lines connecting these points were assumed as a background signal of raw data. The 6 data sets were transformed linearly using a 6 x 6 matrix. This matrix was made in order to transform the 6 data which were obtained by electrophoresis of single dye, into one dominant datum and 5 quite minor data for each dye. Each of the obtained peaks was basecalled as a corresponding base (A,G,C,T,isoG,or isoC) (These techniques are described in Giddings MC, Brumley RL Jr, Haker M, Smith LM., "An adaptive, object oriented strategy for base calling in DNA sequence analysis," *Nucleic Acids Res.* 1993 Sep 25;21(19):4530-40. ; Berno AJ., "A graph theoretic approach to the analysis of DNA sequencing data". *Genome Res.* 1996 Feb;6(2):80-91). Electropherograms (that is a 6-channel chromatogram) of analyzed data are shown in Figures 4 and 5, in which the sequence reported is limited to the portion comprising the included isoG and isoC bases (indicated as lower "g" and "c" letters in the sequence, and "iG" and "iC" in the Figure). The sequence was ATAGTGTCACCTAAAgAcCAAGGCGAgAcCATGGTCATAG (SEQ ID NO:20).

Various articles of the scientific and patent literature are cited throughout this specification. Each such article is hereby incorporated by reference in its entirety and for all purposes by such citation.

## CLAIMS

1. A method for preparing nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases comprising:
- a) providing a specific ligand;
  - 5 b) synthesizing a pool of nucleic acid aptamers comprising at least one base capable of base pairing and different from the standard W-C bases;
  - c) mixing the pool of aptamers with the specific ligand;
  - d) selecting and amplifying a specific aptamer that binds to the specific ligand.
2. The method of claim 1, wherein the aptamer comprises at least one standard W-C base
- 10 A, C, G, T or U and at least one base capable of base pairing and different from the standard W-C bases.
3. The method of claim 1, wherein the base capable of base pairing and different from the W-C standard bases is selected from the group consisting of: iso-C; iso-G; 2,6-diaminopyrimidine; xanthine; 6-amino-5-substituted pyrazin-2(1H)-one;
- 15 1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione;
- 5-aza-7-deazaguanine; 6-amino-3-substituted pyrazin-2(1H)-one;
- 3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine;
- 2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one; 3-methyl isocarbostyryl;
- 5-methyl isocarbostyryl; 7-propynyl isocarbostyryl; m-xylene;
- 20 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylnaphthalene;
- 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl;
- 6-methyl-7-azaindole; 3-propynyl-7-azaindole; imidazopyridine; pyrrolopyridine;
- and a heterocyclic base having the structural formula



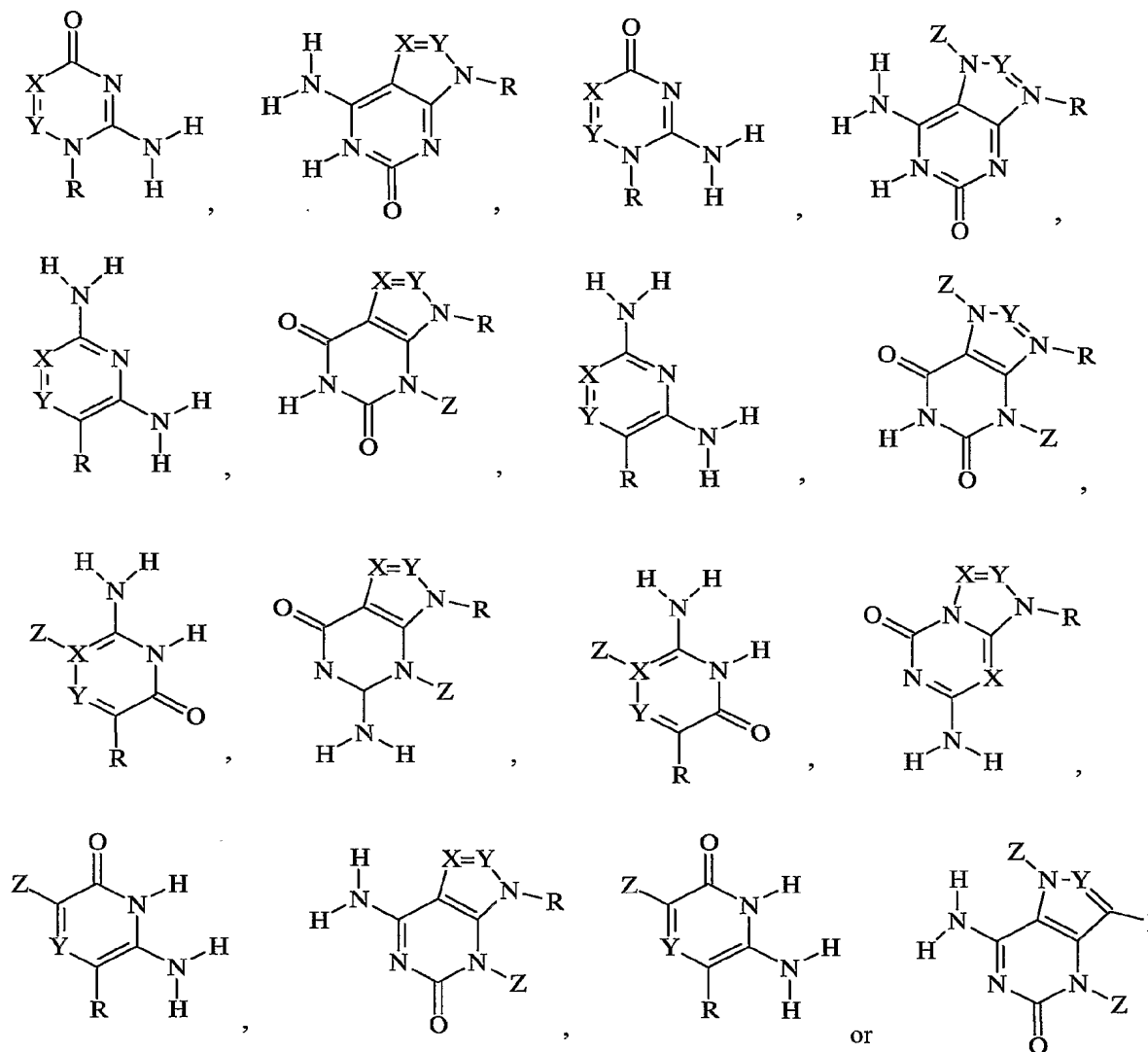
wherein R designates the point of attachment of the base to position 1 of ribose or deoxyribose ring, X is either a nitrogen atom or a carbon atom bearing a substituent Z. Z is either a hydrogen, an unfunctionalized lower alkyl chain, or a lower alkyl chain bearing an amino, carboxyl, hydroxyl, thiol, aryl, indole, or imidazolyl group, Y is either N or CH, and each ring contains no more than three nitrogens consecutively bonded.

4. The method of claim 1, wherein the at least one base capable of base pairing and different from the standard W-C bases is isoC and/or isoG.

10 5. The method of any one of claims 1-4, wherein the amplification of step d) is performed

by polymerase chain reaction and wherein the base triphosphates different from W-C bases have a different concentration from the concentration of the standard W-C base triphosphates.

6. The method of any one of claims 1-4, wherein the amplification of step d) is performed by polymerase chain reaction and wherein a base different from the standard W-C bases is isoC and/or isoG and the concentration of isoC and isoG triphosphate is lower than the concentration of the standard W-C bases triphosphates.
7. The method of claim 1, wherein the ligand is selected from the group consisting of amino acids, peptides, proteins, lipids, oligosaccharides, alkaloids, terpenes, co-enzymes, antibiotics, and their derivatives and their complexes.
8. The method of claim 1, wherein the ligand is a protein or a modified protein.
9. The method of claim 1, wherein the molecular weight of the ligand is less than 5,000 daltons.
10. An isolated aptamer comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases.
11. The aptamer of claim 10, wherein the aptamer comprises at least one standard W-C base A, C, G, T or U and at least one base capable of base pairing and different from the standard W-C bases.
12. The aptamer of claim 10, wherein said base different from the standard W-C bases is selected from the group consisting of: iso-C; iso-G; 2,6-diaminopyrimidine; xanthine; 6-amino-5-substituted pyrazin-2(1H)-one; 1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione; 5-aza-7-deazaguanine; 6-amino-3-substituted pyrazin-2(1H)-one; 3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine; 2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one; 3-methyl isocarbostyryl; 5-methyl isocarbostyryl; 7-propynyl isocarbostyryl; m-xylene; 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylanaphthalene; 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl; 6-methyl-7-azaindole; 3-propynyl-7-azaindole; imidazopyridine; pyrrolopyridine and a heterocyclic base having a structural formula



wherein R designates the point of attachment of the base to position 1 of a ribose or deoxyribose ring, X is either a nitrogen atom or a carbon atom bearing a substituent Z. Z is either a hydrogen, an unfunctionalized lower alkyl chain, or a lower alkyl chain bearing an amino, carboxyl, hydroxyl, thiol, aryl, indole, or imidazolyl group, Y is either N or CH, and each ring contains no more than three nitrogens consecutively bonded.

13. The aptamer of claim 10, wherein said base capable of base pairing and different from the standard W-C bases is isoC and/or isoG.

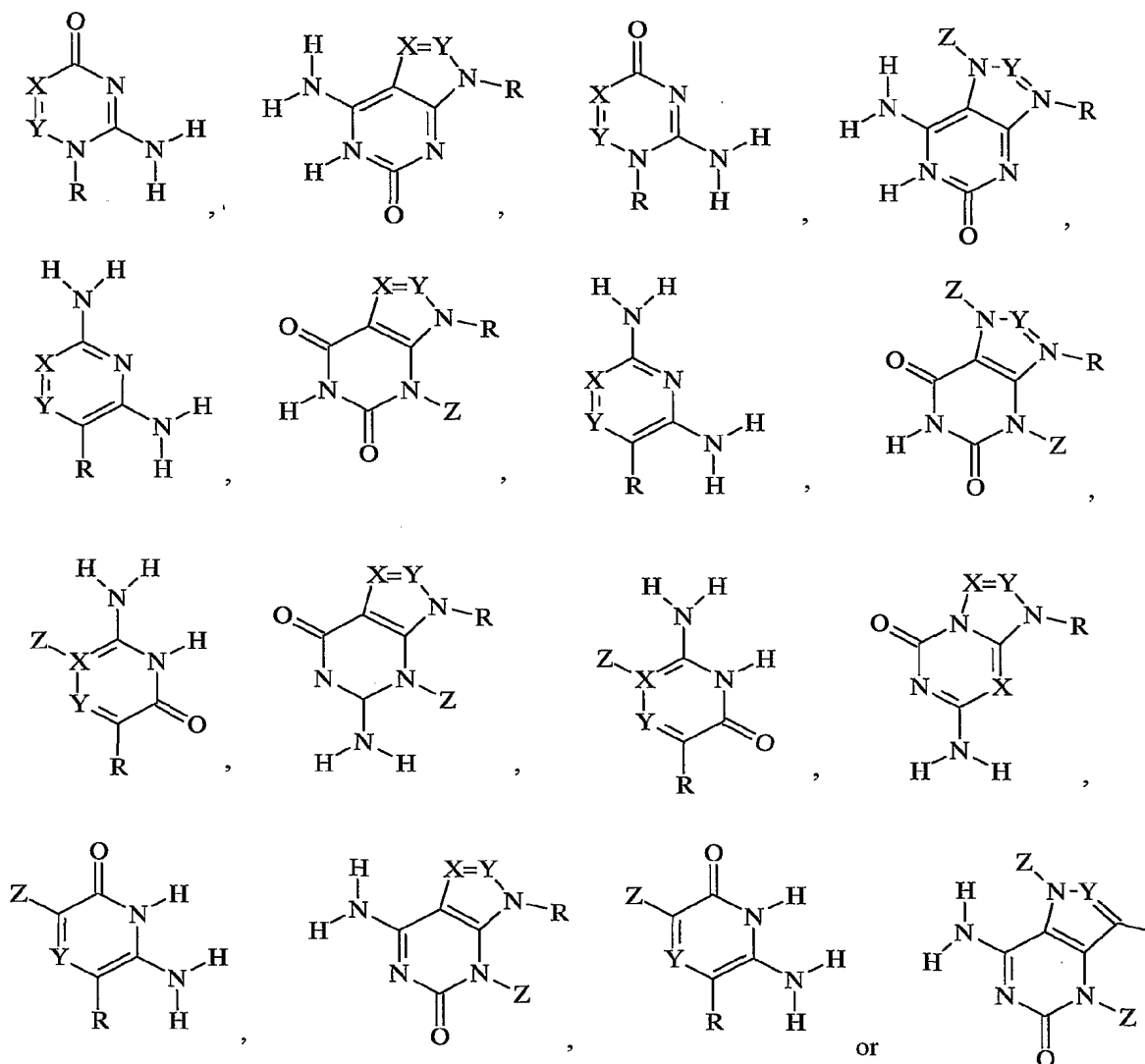
10 14. The aptamer of claim 10, which is a single, double or triple stranded nucleic acid.



15. The aptamer of any one of claims 10-14, which is DNA, RNA or PNA.
16. The aptamer of any one of claims 10-15, wherein the number of bases of the aptamer is 1-300.
17. A method for isolating a specific ligand from a pool of ligands comprising:
  - 5     i) providing at least one specific aptamer according to one of claims 10-16;
  - ii) mixing the aptamer with a pool of ligands; and
  - iii) recovering the specific ligand bound to the specific aptamer.
18. The method of claim 17, wherein the ligand is selected from the group consisting of amino acids, peptides, proteins, lipids, oligosaccharides, alkaloids, terpenes,  
10     co-enzymes, antibiotics, and their derivatives and their complexes.
19. The method of claim 17, wherein the ligand is a protein or a modified protein.
20. The method of claim 19, wherein the protein is phosphorylated .
21. The method of claim 17, wherein the aptamer discriminates between phosphorylated and non-phosphorylated ligand proteins.
- 15   22. The method of claim 19, wherein the protein is glycosylated .
23. The method of claim 17, wherein the aptamer discriminates between glycosylated and non-glycosylated ligand proteins.
24. The method of claim 17, wherein the aptamer is fixed on an insoluble substrate.
25. The method of claim 24, wherein the aptamer is fixed on an insoluble substrate by  
20     means of a spacer sequence.
26. The method of claim 24 or 25, wherein the insoluble substrate is a chip substrate.
27. The method of claim 17, wherein the pool of ligand is labeled.
28. An insoluble substrate having at least one nucleic acid aptamer according to any one of claims 10-16 fixed thereto.
- 25   29. The insoluble substrate of claim 28, wherein the aptamer is fixed to the substrate by means of a spacer sequence.
30. The insoluble substrate of claim 28, comprising a plurality of aptamers that specifically bind to particular known ligands.
31. The insoluble substrate of any one of claims 28-30 that is a chip substrate.
- 30   32. A method for the detection of specific ligand from a biological sample, comprising:
  - I) selecting at least one specific aptamer, according to any one of claims 10-16, capable of binding to a specific ligand from a biological sample;

- II) mixing the at least one the specific aptamer with a biological sample to allow binding of the ligand to the at least one aptamer;
- III) detecting the presence and/or quantity of the specific ligand from the biological sample bound to the at least one aptamer.
- 5 33. The method of claim 32, wherein the aptamer is in solution or fixed to a substrate.
34. The method of claim 32, wherein the ligand in the biological sample is labeled, and the presence and/or quantity of the specific ligand is determined by detecting the label.
35. The method of claim 32, wherein the method is a diagnostic method and a diagnosis  
10 of a disease is based upon the amount or presence or absence of the ligand bound to the aptamer.
36. A detection or diagnostic kit for the determination of a specific ligand selected from a pool of ligands comprising at least one specific aptamer according to any one of claims 10-16, or at least one substrate having one or more aptamers according to any  
15 one of claims 25-31 fixed on it.
37. The kit of claim 36, further comprising one or more labels for labelling a pool of ligands.
38. A pharmaceutical composition comprising the aptamer according to any one of claims 10-16 and a pharmaceutically suitable diluent, excipient and/or carrier.
- 20 39. A method for determining the nucleotide base sequence of a nucleic acid template comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases comprising:
- a) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- 25 b) elongating said template using a primer or a promoter or a promoter and an initiator in the presence of a nucleic acid synthesizing enzyme, nucleic acid synthesizing enzyme substrates and nucleic acid enzyme substrate derivatives;
- c) determining the base sequence of the template as the reverse complement of the sequence of the elongation product obtained in b).
- 30 40. The method of claim 39, wherein the product of step b) comprises a plurality of polynucleotides and each of said polynucleotides is separated from the others differing in length by a single nucleotide.

41. The method of claim 39, wherein after step a) an annealing reaction between said template and a primer able to hybridize to said template is carried out.
42. The method of claim 41, wherein the nucleic acid template comprises at least one standard W-C base A, C, G, T or U and at least one base capable of base pairing and  
5 different from the standard W-C bases.
43. The method of claim 42, wherein the base triphosphates different from the standard W-C bases are present at a different concentration than the standard W-C base triphosphates are present at in the elongation step b).
44. The method of claim 39, wherein the base capable of base pairing and different from  
10 the standard W-C bases is selected from the group consisting of: iso-C; iso-G; 2,6-diaminopyrimidine; xanthine; 6-amino-5-substituted pyrazin-2(1H)-one; 1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione; 5-aza-7-deazaguanine; 6-amino-3-substituted pyrazin-2(1H)-one; 3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine;  
15 2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one; 3-methyl isocarbostyryl; 5-methyl isocarbostyryl; 7-propynyl isocarbostyryl; m-xylene; 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylnaphthalene;  
20 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl; 6-methyl-7-azaindole; 3-propynyl-7-azaindole; imidazopyridine; pyrrolopyridine and a heterocyclic base having a structural formula of



wherein R designates the point of attachment of the base to position 1 of a ribose or deoxyribose ring, X is either a nitrogen atom or a carbon atom bearing a substituent Z. Z is either a hydrogen, an unfunctionalized lower alkyl chain, or a lower alkyl chain bearing an amino, carboxyl, hydroxyl, thiol, aryl, indole, or imidazolyl group, Y is either N or CH, and each ring contains no more than three nitrogens consecutively bonded.

45. The method of claim 39, wherein the base capable of base pairing and different from the standard W-C bases is isoC and/or isoG.

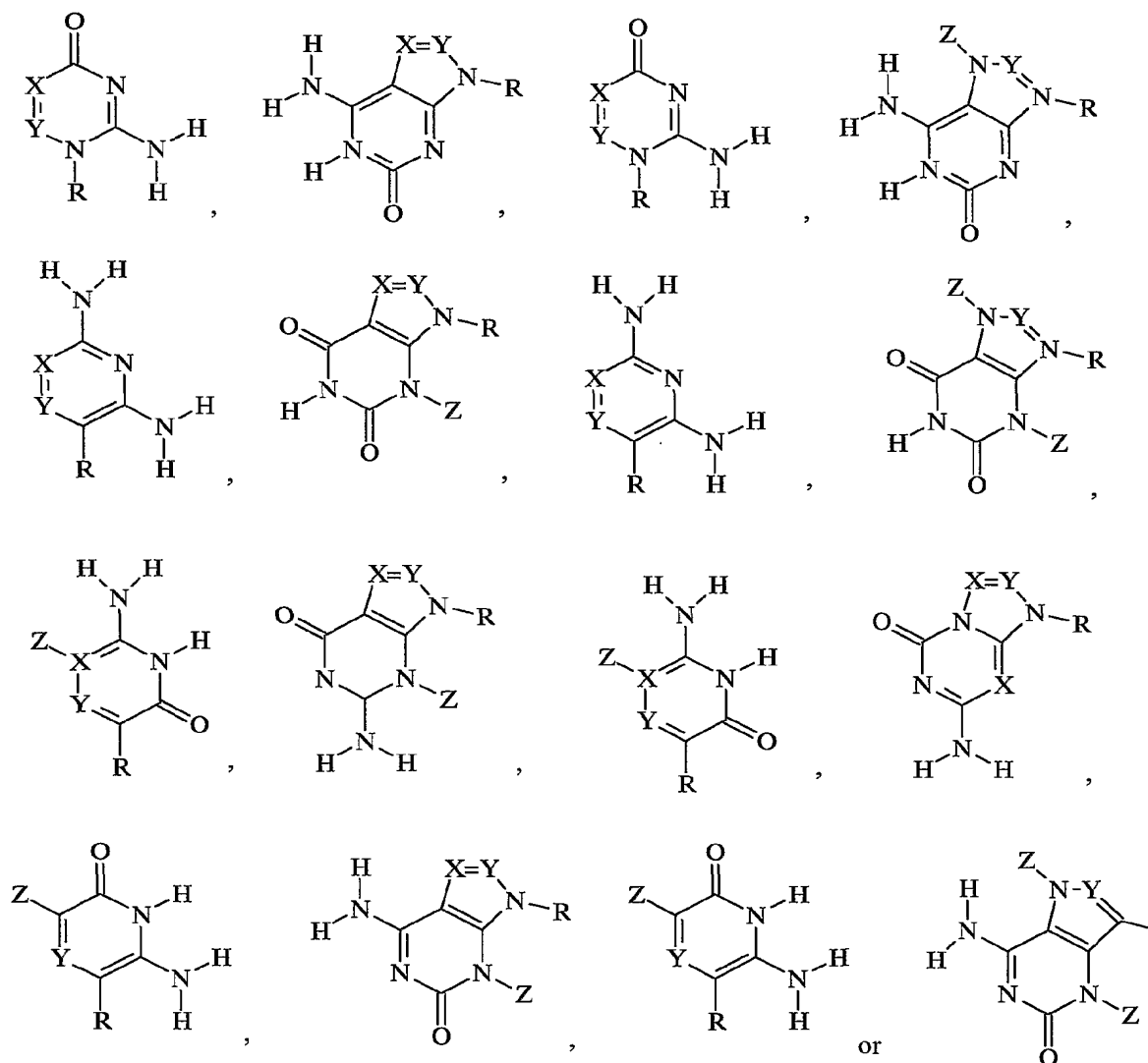
10 46. The method of claim 45, wherein the concentration of isoC and isoG triphosphate is

lower than the concentration of the standard base triphosphates in the elongation step b).

47. The method of claim 37, wherein the template is DNA, the synthesizing enzyme is DNA-dependent DNA polymerase, the synthesizing enzyme substrate is a plurality of dNTPs and substrate derivatives comprise ddNTPs.
48. The method of claim 47, wherein a dNTP or a ddNTP is labeled.
49. The method of claim 48, wherein the primer or initiator is labeled.
50. The method of claim 48 or 49, wherein the label is an isotope, a chromophore or a fluorophore.
51. The method of claim 39, wherein the template is DNA and the synthesizing enzyme is a DNA-dependent RNA polymerase, the synthesizing enzyme substrate is a plurality of NTPs and substrate derivatives comprise 3'-dNTPs derivatives.
52. The method of claim 51, wherein a NTP or a 3'-dNTP derivative is labeled.
53. The method of claim 51, wherein the primer or initiator is labeled.
54. The method of claim 52 or 53, wherein the label is an isotope, a chromophore or a fluorophore.
55. The method of claim 39, wherein the template is RNA and the synthesizing enzyme is a RNA-dependent DNA polymerase, the synthesizing enzyme substrate is a plurality of dNTPs and the substrate derivatives comprise ddNTPs.
56. The method of claim 55, wherein a dNTP or a ddNTP is labeled.
57. The method of claim 56, wherein the primer or initiator is labeled.
58. The method of claim 56 or 57, wherein the label is an isotope, a chromophore or a fluorophore.
59. The method of claim 39, wherein the template is RNA and the synthesizing enzyme is a RNA-dependent RNA polymerase, the synthesizing enzyme substrate is a plurality of NTPs and the substrate derivatives comprise 3'-dNTPs derivatives.
60. The method of claim 59, wherein a NTP or a 3'-dNTP derivative is labeled.
61. The method of claim 59, wherein the primer or initiator is labeled.
62. The method of claim 60 or 61, wherein the label is an isotope, a chromophore or a fluorophore.
63. A method for determining the nucleotide base sequence of a nucleic acid template comprising at least one base capable of base pairing and different from the standard

Watson-Crick (W-C) bases comprising:

- a) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
  - b) labeling one end of said template;
  - 5 c) chemically degrading said labeled template;
  - d) determining the length of the products obtained in c, obtaining the sequence of the template as the sequence of the incremental lengths of the products.
64. The method of claim 63, wherein the nucleic acid template comprises at least one standard W-C base and at least one base capable of base pairing and different from the standard W-C bases.
- 10 65. The method of claim 63, wherein the base capable of base pairing and different from the standard W-C bases is selected from the group consisting of: iso-C; iso-G; 2,6-diaminopyrimidine; xanthine; 6-amino-5-substituted pyrazin-2(1H)-one;
- 15 1-methyl-pyrazolo[4,3-d]pyrimidine-5,7(4H,6H)-dione; 5-aza-7-deazaguanine; 6-amino-3-substituted pyrazin-2(1H)-one;
- 3-amino-1-methylpurin-2-one; 2,4-diamino-5,6-dihydropyrimidine;
- 2-amino-6-(N,N-dimethylamino)purine; pyridin-2-one;
- 3-methyl isocarbostyryl; 5-methyl isocarbostyryl; 7-propynyl
- 20 isocarbostyryl; m-xylene; 1,3,4-trimethylbenzene; 2-methylanaphthalene; 1,4-dimethylnaphthalene;
- 1-methylanaphthalene; naphthalene; 7-azaindole; isocarbostyryl;
- 6-methyl-7-azaindole;
- 3-propynyl-7-azaindole; imidazopyridine; and pyrrolopyridine and having a
- 25 hetrocyclic base having a structural formula of



wherein R designates the point of attachment of the base to position 1 of a ribose or deoxyribose ring, X is either a nitrogen atom or a carbon atom bearing a substituent Z. Z is either a hydrogen, an unfunctionalized lower alkyl chain, or a lower alkyl chain bearing an amino, carboxyl, hydroxyl, thiol, aryl, indole, or imidazolyl group, Y is either N or CH, and each ring contains no more than three nitrogens consecutively bonded.

66. The method of claim 63, wherein the base capable of base pairing and different from the standard W-C bases is isoC and/or isoG.

10 67. A method for the determination of the base sequence of a nucleic acid template

comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases,:

- A) providing a nucleic acid template comprising at least one base capable of base pairing and different from the standard W-C bases;
- 5 B) elongating said template using a primer or a promoter or a promoter and an initiator in the presence of a nucleic acid synthesizing enzyme, nucleic acid synthesizing enzyme substrates and nucleic acid synthesizing enzyme substrate derivatives;
- C) determining the base sequence of the elongation product obtained in
- 10 B) using MALDI-TOF-MS analysis.

68. A method for the determining the base sequence of a nucleic acid template comprising at least one base capable of base pairing and different from the standard Watson-Crick (W-C) bases,:

- a) providing a nucleic acid template comprising at least one base capable of
- 15 base pairing and different from the standard W-C standard bases;
- b) carrying out elongation by using a primer, a promoter, or a promoter and initiator in the presence of nucleoside triphosphates whose base comprises at least one base capable of base pairing and different from the standard W-C bases by adding a first nucleoside triphosphate and detecting PPi release and
- 20 degrading the first nucleoside triphosphate, then adding a second nucleoside triphosphate and detecting PPi and degrading the second nucleoside triphosphate, and repeating the procedure according to the kind of base of the nucleoside triphosphates provided;
- c) repeating step b) above according to the template bases number desired to be
- 25 sequenced;
- d) determining the sequence of the template as the sequence of the nucleoside triphosphates added.

69. A method for determining the base sequence of a nucleic acid template comprising at least one base capable of base pairing and different from the standard Watson-Crick

30 (W-C) bases,:

- a) providing a plurality of oligonucleotides fixed on a chip, the oligonucleotides comprising at least one base capable of base pairing and



different from the standard W-C bases and having overlapping frames displaced by one or two bases;

b) hybridizing the oligonucleotides with a labeled template according to the invention comprising at least one base capable of base pairing and different from the standard W-C bases;

c) detecting the signal of the label;

d) determining the sequence of the template as the set of overlapping oligonucleotides that are labeled.

70. A compound 2',3'-dideoxyisoguanosine 5'-triphosphate (ddisoG) or 2',3'-dideoxyisocytidine 5'-triphosphate (ddisoC).

71. A method for the preparation of 2',3'-dideoxyisoguanosine 5'-triphosphate comprising:

preparing the N-oxide of ddATP by precipitation of a mixture of disodium salt of ddATP and a solution of monopermaleic acid;

purifying the prepared N-oxide of ddATP and irradiating the purified N-oxide of ddATP with light from a high pressure mercury arc lamp; and recovering the final product 2',3'-dideoxyisoguanosine 5'-triphosphate.

72. A method for the preparation of 2',3'-dideoxyisoguanosine 5'-triphosphate comprising:

adding hydrogen peroxide to a solution of sodium carbonate in water;

adding maleic anhydride and stirring the mixture until all of the maleic acid is dissolved;

adding concentrated sulfuric acid in water at 0°C;

extracting the mixture with ether;

obtaining a solution of monopermaleic acid by evaporating the ether extract in the presence of water in a stream of air;

adjusting the pH of the solution 7.0 with hydroxide ion;

adding a solution of disodium salt of ddATP;

stirring the reaction mixture at room temperature;

adjusting the pH to 4.5;

adding absolute EtOH;

recovering the resulting precipitate by centrifugation;

- dissolving the precipitate in water;  
adjusting the pH to 4.5;  
recovering the N-oxide of ddATP by precipitation with ether;  
dissolving the N-oxide of ddATP in water;  
5 placing the solution of the N-oxide of ddATP in a photochemical reaction tube  
and irradiating the solution;  
adjusting the pH to 10;  
stirring the solution at room temperature;  
removing the water; and  
10 purifying the product by HPLC.
73. A method for preparing 2',3'-dideoxyisocytidine 5'-triphosphate comprising:  
preparing 2',3'-deoxyisocytidine from a mixture of  
2,5'-anhydro-2',3'-dideoxyuridine and methanol;  
adding a solution of triethylammonium bicarbonate to 2',3'-deoxyisocytidine and  
15 removing the solvent by evaporation;  
purifying the product obtained above by HPLC; and  
recovering 2',3'-dideoxyisocytidine 5'-triphosphate.
74. A method for preparing 2',3'-dideoxyisocytidine 5'-triphosphate comprising:  
adding diethyl azodicarboxylate to a mixture of 2',3'-dideoxyuridine and  
20 triphenylphosphine suspended in tetrahydrofuran;  
stirring the mixture at room temperature overnight to obtain a pale yellow  
suspension containing 2,5'-anhydro-2',3'-dideoxyuridine;  
adding the suspension of 2,5'-anhydro-2',3'-dideoxyuridine to methanol  
saturated with dry ammonia;  
25 stirring the mixture at room temperature;  
removing the solvent ;  
extracting the residue with water;  
evaporating the water to obtain 2',3'-deoxyisocytidine;  
treating a mixture of 2',3'-deoxyisocytidine in trimethyl phosphate with  
30 phosphoryl chloride at 0°C ;  
adding a solution of tris(tributylammonium) pyrophosphate in  
dimethylformamide to the mixture and agitating vigorously;

adding a solution of triethylammonium bicarbonate;  
removing the solvents; and  
purifying the product by HPLC.

Fig. 1

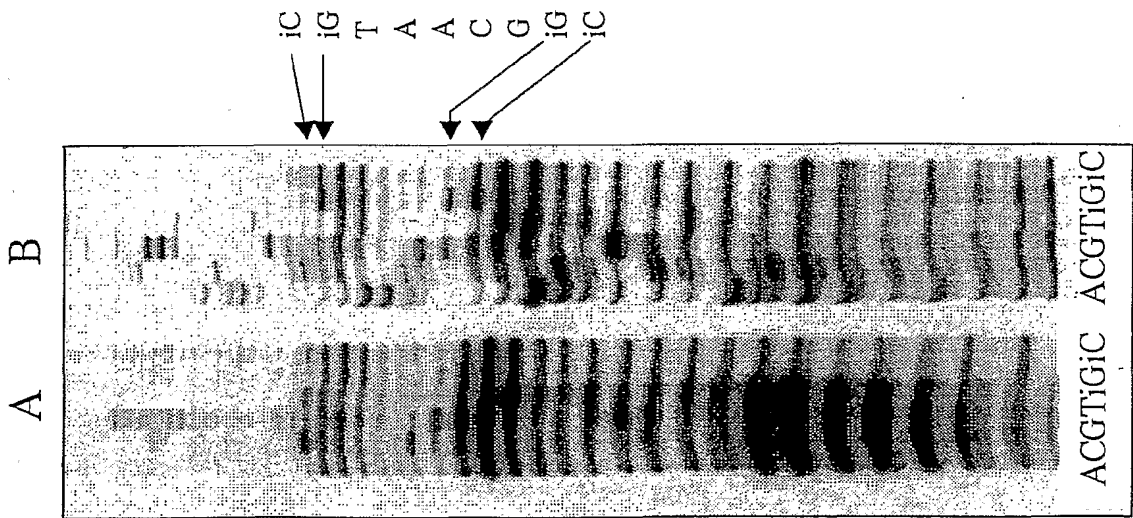
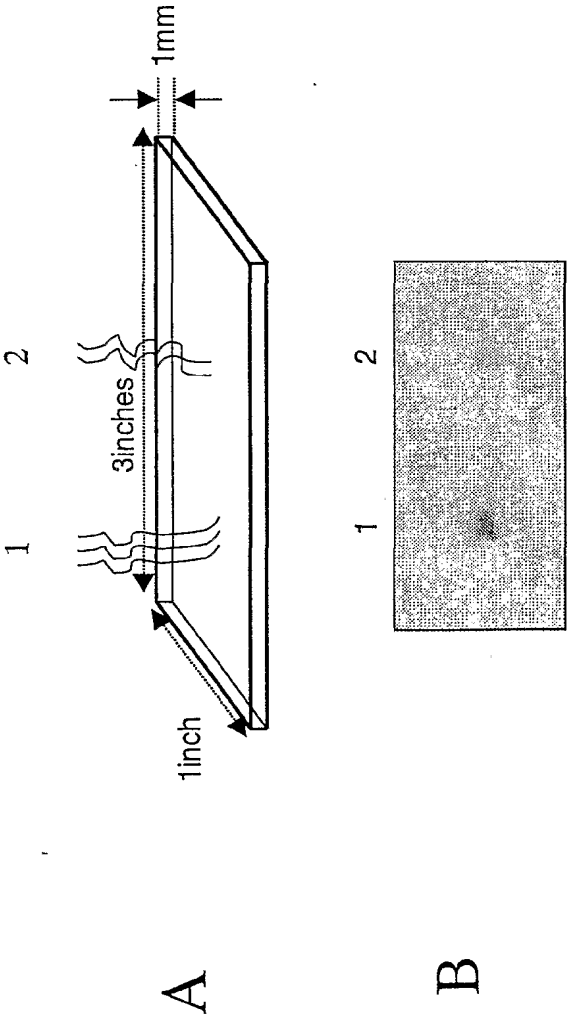


Fig. 2.



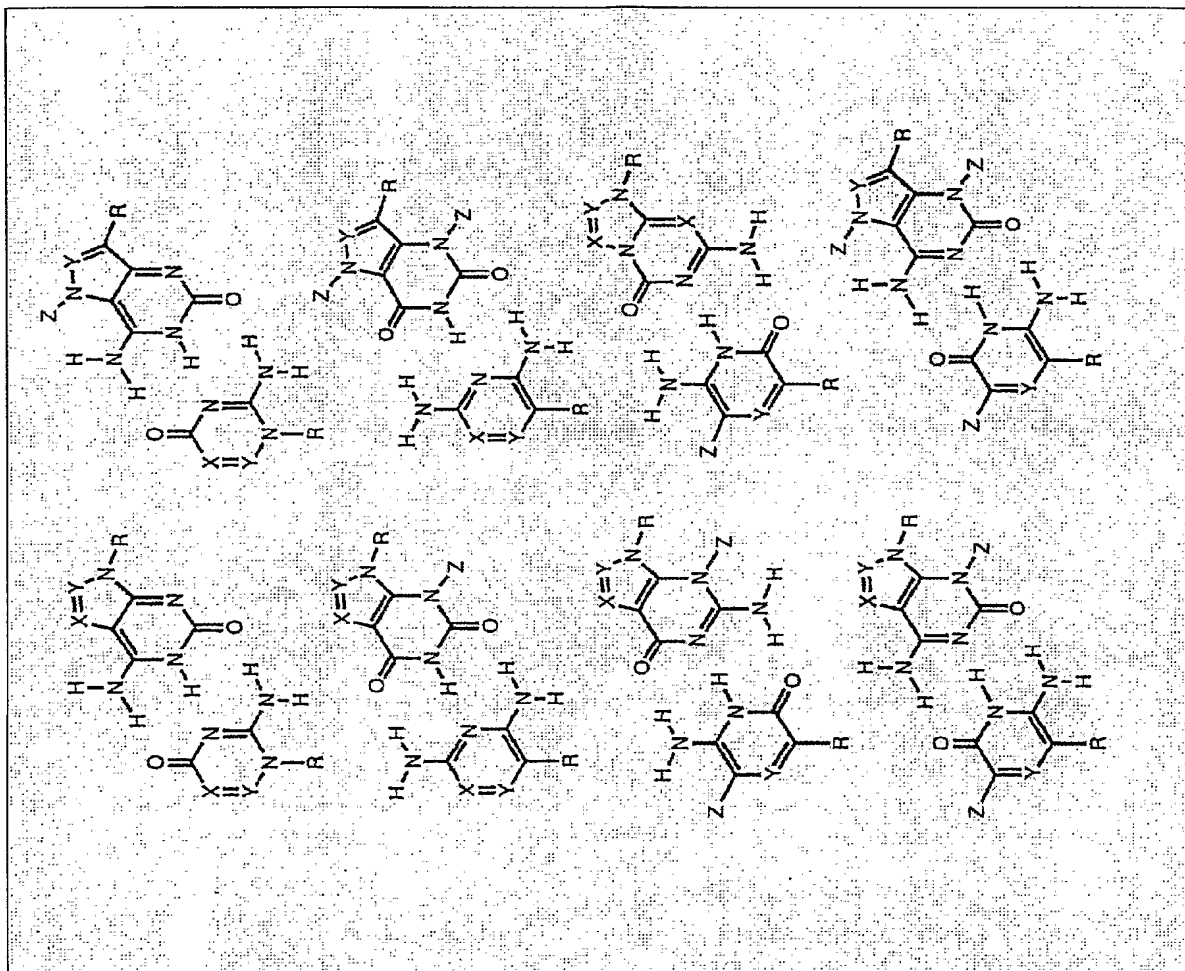


Fig. 3

Fig. 4

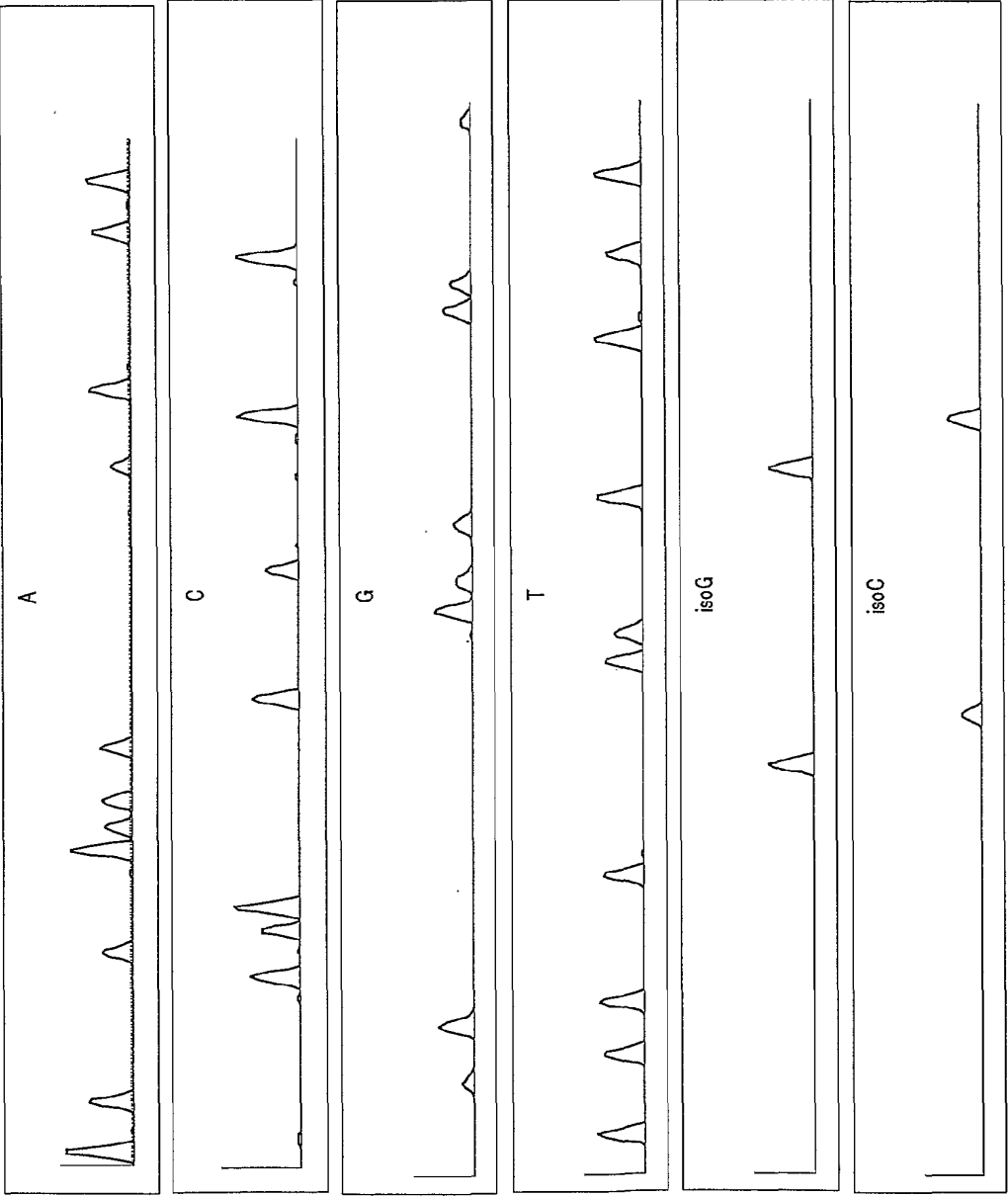
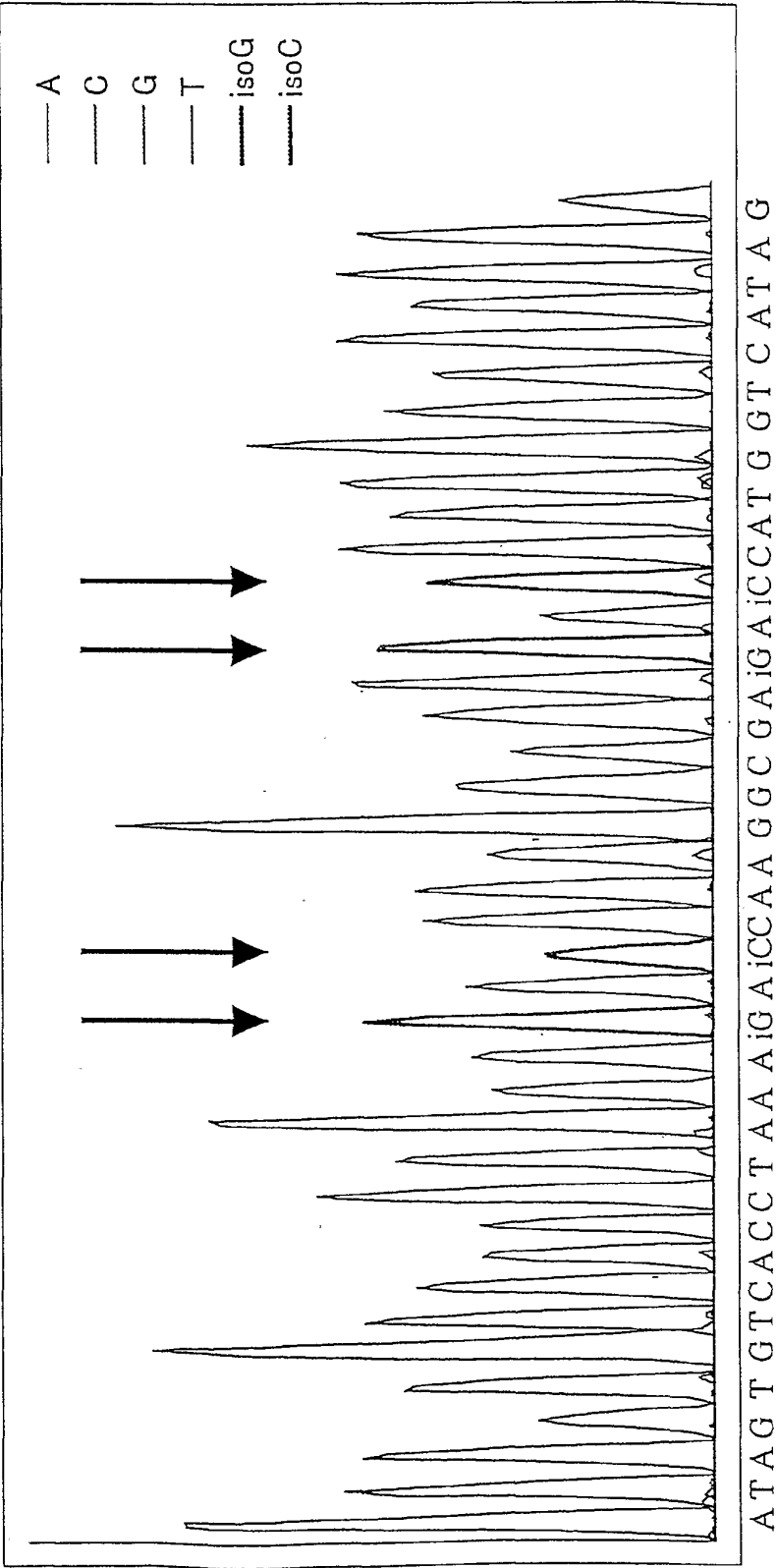


Fig. 5



(d/ddNTP,d/ddisoG,d/ddisoC)



1/8

## SEQUENCE LISTING

&lt;110&gt; RIKEN

HAYASHIZAKI, Yoshihide

<120> METHOD FOR BASE SEQUENCING AND BIOLOGICALLY ACTIVE  
NUCLEIC ACIDS

&lt;130&gt; 1243

&lt;140&gt;

&lt;141&gt;

&lt;150&gt; US 60/253097

&lt;151&gt; 2000-11-28

&lt;160&gt; 17

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: a reverse 3'  
primer

&lt;400&gt; 1

agcggataac aatttcacac

20

&lt;210&gt; 2

&lt;211&gt; 70

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: an  
oligonucleotide template

&lt;400&gt; 2

cacgacgttg taaaacgacg gccagtgtta cggcattgcc gatgacgatg gtgtgaaatt 60  
gttatccgct 70

&lt;210&gt; 3

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&lt;211&gt; 27

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: a sequence  
sequenced in Example 2

&lt;400&gt; 3

ttcacacccat cgtcatcggc aatgccg

27

&lt;210&gt; 4

&lt;211&gt; 1214

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (300)..(935)

&lt;400&gt; 4

gatgttcaag gtgagaatgt ggagccgggt cacagctgtc attgtcccct ctcagcctcc 60

aggagccctc cccacgctcc cgggogtctt tttgcgggac cagactcggt tcctcctgag 120

gccttttttca cctccgggct aaattctggc ttggcggcgg ttcattcagc actcgggtacc 180

agcacctgtg aggttgtgga gtgaaaccct agattggtgg gatcggccct ttgagctctc 240

tcgcgaatga aacactttga aaagttaaaa cgcctctatc gtgccacac cctcgggcta 299

atg ccc ttt tgg ggt tgt ggg gag gat gaa gcc agg tct ggg cgc tgc 347

Met Pro Phe Trp Gly Cys Gly Glu Asp Glu Ala Arg Ser Gly Arg Cys

1

5

10

15

agg gta att cag aga tct gtg ggg cca gcc agc ctg agc ctg ctc acc 395

Arg Val Ile Gln Arg Ser Val Gly Pro Ala Ser Leu Ser Leu Leu Thr

20

25

30

ttc aga gtc tat gca gca ccc aaa aag gac tcg cct cac aaa agt tac 443

Phe Arg Val Tyr Ala Ala Pro Lys Lys Asp Ser Pro His Lys Ser Tyr

35

40

45

atg aag atc gat gag ctt tca ctc tac tca gtt cct gag ggt caa tct 491

Met Lys Ile Asp Glu Leu Ser Leu Tyr Ser Val Pro Glu Gly Gln Ser

50

55

60

3/8

aaa tat gtg gag gag cca agg act caa ctt gaa gaa aac atc tca caa 539  
 Lys Tyr Val Glu Glu Pro Arg Thr Gln Leu Glu Glu Asn Ile Ser Gln  
 65 70 75 80

ctc cga cat cat tgt gag cca tat aca agt ttc tgt cag gaa ata tac 587  
 Leu Arg His His Cys Glu Pro Tyr Thr Ser Phe Cys Gln Glu Ile Tyr  
 85 90 95

tcc cat act aaa ccc aag gtg gat cac ttt gtc cag tgg gga gta gac 635  
 Ser His Thr Lys Pro Lys Val Asp His Phe Val Gln Trp Gly Val Asp  
 100 105 110

aac tat aac tat ctt caa aat gcg cct cct gga ttt ttc cca aga ctc 683  
 Asn Tyr Asn Tyr Leu Gln Asn Ala Pro Pro Gly Phe Phe Pro Arg Leu  
 115 120 125

ggt gtt att ggt ttt gct ggt ttt gtt gga ctc ctt ttt gct aga ggt 731  
 Gly Val Ile Gly Phe Ala Gly Phe Val Gly Leu Leu Phe Ala Arg Gly  
 130 135 140

tca aaa ata aag aag ctg gtg tat cct cct ttt ttc atg gga tta ggt 779  
 Ser Lys Ile Lys Lys Leu Val Tyr Pro Pro Phe Phe Met Gly Leu Gly  
 145 150 155 160

gcc tct gtc tat tac cca caa caa gcc atc acc att gcc cag atc act 827  
 Ala Ser Val Tyr Tyr Pro Gln Gln Ala Ile Thr Ile Ala Gln Ile Thr  
 165 170 175

ggg gag aag tta tat gac tgg gga tta cga ggg tac ata gtt ata gaa 875  
 Gly Glu Lys Leu Tyr Asp Trp Gly Leu Arg Gly Tyr Ile Val Ile Glu  
 180 185 190

gat ttg tgg aag caa aat ttt cag aag cca gga aat gtg aag aat tca 923  
 Asp Leu Trp Lys Gln Asn Phe Gln Lys Pro Gly Asn Val Lys Asn Ser  
 195 200 205

cct gga aat aaa tagaaaactc catgctctgc ccatttcaat cagttatagg 975  
 Pro Gly Asn Lys  
 210

taaacattgg aaacttcaga cagtaaatca gtattttctac agacaaatgg cgaaatcagt 1035

attggatata gtaaactggc tttcttcagg aaaaacaaca ctaagccttt ttgctctttt 1095

gggatgatgcc atattacagg ccaactaato tgcaatcttt cacatggaaa taatgtacaa 1155

gccttagaac tcttcattct tataccacta tttatgtaca taattaaact ccagattcc 1214

4/8

&lt;210&gt; 5

&lt;211&gt; 212

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 5

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Pro | Phe | Trp | Gly | Cys | Gly | Glu | Asp | Glu | Ala | Arg | Ser | Gly | Arg | Cys |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     |     | 15  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Arg | Val | Ile | Gln | Arg | Ser | Val | Gly | Pro | Ala | Ser | Leu | Ser | Leu | Leu | Thr |
|     |     |     | 20  |     |     |     |     | 25  |     |     |     |     | 30  |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Phe | Arg | Val | Tyr | Ala | Ala | Pro | Lys | Lys | Asp | Ser | Pro | His | Lys | Ser | Tyr |
|     |     | 35  |     |     |     |     | 40  |     |     |     |     | 45  |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Lys | Ile | Asp | Glu | Leu | Ser | Leu | Tyr | Ser | Val | Pro | Glu | Gly | Gln | Ser |
|     | 50  |     |     |     |     | 55  |     |     |     |     | 60  |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Lys | Tyr | Val | Glu | Glu | Pro | Arg | Thr | Gln | Leu | Glu | Glu | Asn | Ile | Ser | Gln |
| 65  |     |     |     |     | 70  |     |     |     |     | 75  |     |     |     |     | 80  |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Leu | Arg | His | His | Cys | Glu | Pro | Tyr | Thr | Ser | Phe | Cys | Gln | Glu | Ile | Tyr |
|     |     |     |     | 85  |     |     |     |     | 90  |     |     |     |     | 95  |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ser | His | Thr | Lys | Pro | Lys | Val | Asp | His | Phe | Val | Gln | Trp | Gly | Val | Asp |
|     |     |     | 100 |     |     |     |     | 105 |     |     |     |     | 110 |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asn | Tyr | Asn | Tyr | Leu | Gln | Asn | Ala | Pro | Pro | Gly | Phe | Phe | Pro | Arg | Leu |
|     | 115 |     |     |     |     |     | 120 |     |     |     |     | 125 |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Val | Ile | Gly | Phe | Ala | Gly | Phe | Val | Gly | Leu | Leu | Phe | Ala | Arg | Gly |
|     | 130 |     |     |     |     | 135 |     |     |     |     | 140 |     |     |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ser | Lys | Ile | Lys | Lys | Leu | Val | Tyr | Pro | Pro | Phe | Phe | Met | Gly | Leu | Gly |
| 145 |     |     |     |     | 150 |     |     |     |     | 155 |     |     |     | 160 |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Ala | Ser | Val | Tyr | Tyr | Pro | Gln | Gln | Ala | Ile | Thr | Ile | Ala | Gln | Ile | Thr |
|     |     |     |     | 165 |     |     |     |     | 170 |     |     |     |     | 175 |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Gly | Glu | Lys | Leu | Tyr | Asp | Trp | Gly | Leu | Arg | Gly | Tyr | Ile | Val | Ile | Glu |
|     |     |     | 180 |     |     |     |     | 185 |     |     |     |     | 190 |     |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Leu | Trp | Lys | Gln | Asn | Phe | Gln | Lys | Pro | Gly | Asn | Val | Lys | Asn | Ser |
|     |     |     | 195 |     |     |     |     | 200 |     |     |     | 205 |     |     |     |

|     |     |     |     |
|-----|-----|-----|-----|
| Pro | Gly | Asn | Lys |
|     |     |     | 210 |

<210> 6

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a primer

<400> 6

gtgccacact cctcggcata tgccc

25

<210> 7

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a primer

<400> 7

tgaagtttcc aatgggatcc tataac

26

<210> 8

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a left  
flanking region

<400> 8

tgtaaaacga cggccagt

18

<210> 9

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a right  
flanking region

<400> 9  
gtgtgaaatt gttatccgct

20

<210> 10  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: a primer

<400> 10  
tgtaaaacga cggccagt

18

<210> 11  
<211> 20  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: complement of  
the 3' primer

<400> 11  
gtgtgaaatt gttatccgct

20

<210> 12  
<211> 81  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: a sequence of  
Template 1

<400> 12  
tgtaaaacga cggccagtgc gtaacgggggt ctatgttccc gcacaccgtg gcaaaaactgt 60  
gtgaaattgt tatccgctgc t 81

<210> 13  
<211> 40  
<212> DNA  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a sequence of  
the aptamer portion

<400> 13

gcgtaacggg gtctatgttc ccgcacaccg tggcaaaact

40

<210> 14

<211> 94

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a primer

<400> 14

tgccatttca ttacctcttt ctccgcaccc gacatagatg acactactac ggtatgatcc 60  
tatggagaac gtcagcgga taacaatttc acac 94

<210> 15

<211> 50

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a spacer

<400> 15

gcacccgaca tagatgacac tactacggta tgatcctatg gagaacgctc

50

<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: the annealing  
site to single strand DNA

<400> 16

aggggataac aatttcacac

20

<210> 17

<211> 78

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: a negative  
control aptamer

<400> 17

tgtaaaacga cggccagttt ccggagtcac ggctgcgggc cgtctgagcc gtttgcaagt 60  
gtgaaattgt tatccgct 78